Regulation of protease production in verticillium lecanii

Bye, Natasha Jane

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Regulation of protease production in *Verticillium lecanii*

Submitted by Natasha Jane Bye
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"The outcome of any serious research can only be to make two questions grow where one question grew before"

Thorstein Veblen (1857-1929)
American Economist

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ABBREVIATIONS

A	Absorbance
Ala-	Alanine
AMC	Amino methyl-coumarin
areA	Gene encoding AREA
AREA	Major nitrogen regulatory protein of *Aspergillus nidulans*
Arg-	Arginine
BSA	Bovine Serum Albumin
Bz-	Benzoyl
-C	Carbon derepressed (refers to culture conditions)
+C	Carbon repressed (as above)
°C	Degrees Celsius
CAPS	3-[cyclohexylamino]-1-propanesulphonic acid
c ml^{-1}	Conidia per millilitre
creA	Gene encoding CREA
CREA	Major carbon regulatory protein of *Aspergillus nidulans*
d	Day
Da	Daltons
DAB	Diaminobenzidine
d.f	Degrees of freedom
DFP	Diisopropylfluorophosphate
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dtx	Destruxin
EDTA	Ethylenediaminetetraacetic acid
g	Gram
GlcNAg	N-acetylglucosamine
GlcNAg-ase	N-acetylglucosaminidase
Gly	Glycine
h	Hour

HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IPM	Integrated pest management
kat	Katal
K_m	Binding constant
KOH	Potassium hydroxide
l	Litre
LT_{50}	Time taken for 50% of infected insects to die (lethal time)
M	Molar
m	Metre
mA	Milli-ampere
MES	2-[N-morpholino]ethanesulphonic acid
min	Minute
MOPS	3,-[N-morpholino]propanesulphonic acid
Mr	Molecular weight
mRNA	Messenger RNA
-N	Nitrogen derepressed (refers to culture conditions)
+N	Nitrogen repressed (as above)
NA	Nitroalanine
OD	Optical density
p	Probability
PA	Pascal
PBS	Phosphate buffered saline
PC	Personal computer
PEG	Polyethylene glycol
pI	Isoelectric point
PIPES	Piperazine-N-N'-bis[2-ethanesulphonic acid]
po	Phenoloxidase
ppo	Prophenoloxidase
Phe-	Phenylalanine
PMSF	Phenyl methyl sulphonyl fluoride
Pro-	Proline

PR1	Chymoelastase produced by *Metarhizium anisopliae*
pr1	Gene encoding for PR1
PR2	Trypsin produced by *M.anisopliae*
PR3	Trypsin produced by *M.anisopliae*
PR4	Cysteine protease produced by *M.anisopliae*
PRB1	Basic proteinase produced by *Trichoderma harzianum*
PTU	Phenylthiolurea (anti-clotting agent).
RH	Relative Humidity
RNA	Ribonucleic acid
rpm	Rotations per minute
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis
Suc-	Succinyl
TBS	Tris buffered saline
TBST	Tris buffered saline supplemented with Tween20
TEW	Turkey egg white inhibitor
TM	Trade mark
Tris	N-Tris[hydroxymethyl]methylglycine
UV	Ultra-violet light
V	Volts
Val-	Valine
VCP1	Chymoelastase produced by *Verticillium chlamydosporium*
V_m	Theoretical maximum rate of enzymatic reaction
W	Watts
w/v	Weight/volume

ABSTRACT

An extensive body of evidence has shown that a subtilisin proteinase with chymoelastase-like specificity (PR1) produced by the entomopathogenic fungus *Metarhizium anisopliae*, makes an important contribution to the penetration of host cuticle. The aim of this project was to establish the role of an analogous enzyme produced by the insect pathogen *Verticillium lecanii*. 5 isolates of *V.lecanii* produced several isoforms of a PR1-like enzyme when grown on insect cuticle as the sole source of carbon and nitrogen. The control for most isolates was constitutive production plus enhancement/induction by insect cuticle and repression by low molecular weight carbon containing compounds. Isoforms of PR1 appear to be under different regulatory control, however, within and between isolates.

In addition to insect cuticle, deproteinised chitin also enhanced PR1 production by the aphid derived isolate Vertalec (KV71). Bovine Serum Albumin (BSA) was moderately inductive, however, feeding of gelatin and xylan to derepressed established mycelium did not enhance PR1 activity. A similar level of induction by chitin was observed for all other aphid derived isolates tested. Slow release of N-acetyl glucosamine (GlcNAg), the monomer of chitin, exhibited a similar level of induction to de-proteinised chitin itself. In contrast, the whitefly isolate, Mycotal (KV01) was principally induced by BSA, and no PR1-like activity was detected in GlcNAg cultures. For both Vertalec and Mycotal isolates, the maximum number of isoforms of chymotrypsin was observed on insect cuticle as the sole source of carbon and nitrogen, no additional forms were observed on any other substrate. Vertalec produced one

form that was specifically induced by GlcNAg and one form only produced on cuticle. Mycotal produced two forms on BSA that were not found in chitin or control cultures. No differences in isoform profile were observed between aphid or locust cuticle cultures for either isolate, however, isoforms of proteinase from aphid derived isolates were more efficient at digesting aphid cuticle than locust cuticle.

For most isolates of *V.lecanii*, addition of soluble carbon to cuticle cultures repressed PR1-like activity. Low molecular weight nitrogen was also repressive, but to a lesser extent. Under conditions of nitrogen repression, one form of the enzyme produced by KV71 on cuticle was significantly repressed whereas the others were unaffected. All isoforms were absent under carbon repressive conditions. KV42 appeared to produce different forms of PR1 to KV71 and none were repressed by C or N singly though they were in combination. Enhanced chymotrypsin-like activity was found in the early stages of KV42 infection of peach-potato aphids, *Myzus persicae*, over and above that found in controls and aphids mycosed by isolate KV71. The pIs of key *in vivo* protease isoforms matched those of *in vitro* PR1-like fungal enzymes. Therefore it is suggested that PR1-like enzymes of KV42, which are less subject to catabolite repression, are produced in aphids earlier during mycosis. In support of this contention, polyclonal antibodies raised against major PR1 isoforms from KV71 and KV42 have been used to localise enzymes in sections of infected aphids. The results are discussed in the light of the prospect for the use of mixtures of isolates that have PR1-like enzymes with different regulation for improved mycoinsecticides.

1 General Introduction

1.1. APHIDS AS PESTS - problems for control

Aphids are a common problem in many agroecosystems in both tropical and temperate regions. Many scientists believe that with the advent of global warming resulting in longer growing seasons, pests like aphids will flourish in the United Kingdom in the future, presenting a greater need for improved control (David Nicholson-Lord, 1997). The peach potato aphid, *Myzus persicae* (Hom: Aphididae) is a serious phytophagous pest of World-wide distribution (Devonshire 1989). In addition to causing direct damage to the host plant, it is also a very important vector of plant virus diseases (Buczacki & Harris 1981). *Myzus persicae* has been recorded as transmitting over 100 plant virus diseases, including those of major crops such as sugar beet, sugar cane, potatoes, brassicas, beans, tobacco and citrus (Kennedy et al. 1962). Such plant damage can also decrease the marketability of ornamental crops (Devonshire 1989).

This pale green to yellow aphid (figure 1.1) has been recorded on 305 plant species belonging to 72 families particularly favouring the Compositae and Cruciferae (Tatchell et al. 1982.). Its primary host is peach, however it can also over-winter in temperate regions parthenogenetically on winter-standing Brassicae plants or in stored vegetables (Blackman, 1974).

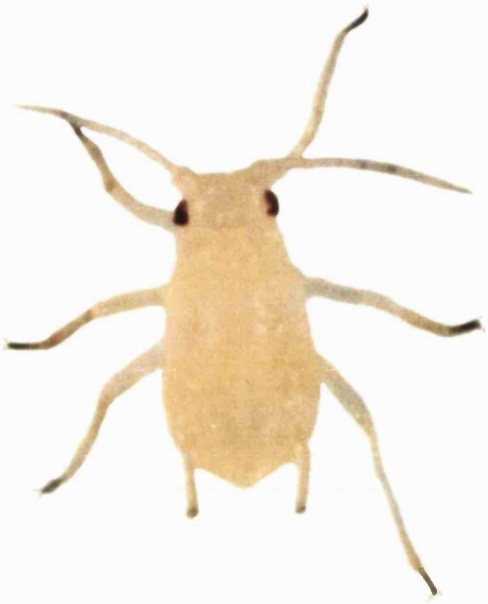


Figure 1.1. *Myzus persicae* - the Peach-Potato Aphid, an important virus vector. (Photograph by Robert Lind, University of Bath).

Chemical insecticides are widely used for pest control, however they may only be temporarily effective due to the advent of resistance (Brattsen 1986). This is particularly true of aphids. High levels of resistance have evolved to many of the insecticides currently used among glasshouse and field populations of *Myzus persicae*.

In addition to the increased costs of finding new replacement products, indiscriminate use of chemicals has led to ecological problems affecting beneficial flora and fauna, and leading to the accumulation of residues in the environment. Recognition of these concerns has led scientists to look for alternative forms of insect control.

A large number of microbial pathogens are known to kill aphids and the observation of natural epizootics in the field have encouraged the development of biopesticides

(Milner 1997). However, for aphid and pest control generally, development of such biological control products has been slow. In 1988, the global insecticide market was $6075 million, only 1% of that being microbial pesticides (Charnley 1991). .

Insect pathogens can be incorporated into control programmes either by small scale release to establish them in the population, or as favoured commercially, they can be applied inundatively as microbial insecticides to bring about short term control (Charnley 1991). It is this latter strategy that is addressed here.

1.2 THE USE OF ENTOMOPATHOGENIC FUNGI TO CONTROL INSECT PESTS

1.2.1. Improving Mycopesticides

Entomopathogenic fungi were among the first organisms to be used for the biological control of pests. Agostino Bassi first enunciated the germ theory of disease when he described the white muscardine disease of silkworms in 1834 (Hall & Papierok 1982). The causative agent, *Beauveria bassiana*, was first recognised as a potential control agent of insect pests in 1874 by Pasteur (cited in Leathers et al. 1993). The first actual control efforts with an entomopathogenic fungus were by Metchnikoff (1879) and Krassilstschik (1888) who attempted to use mass produced *Metarhizium anisopliae* against the wheat cockchafer and sugarbeet curculonid in Russia (cited in Charnley 1989a). Poor trials against a range of species and the advent of chemical pesticides, lead to a loss of interest in microbial control (Charnley 1989a). However, with the

current concerns over chemical products and the increased pressure to find alternatives, microbial control has found renewed appeal.

Insect pathogenic fungi are of great interest to biological control because of their unique mode of infection. They invade their hosts primarily through the cuticle, thus, unlike with entomopathogenic bacteria and viruses, target insects do not need to be actively feeding, allowing the fungus to parasitize pupal and egg stages of many insects. Furthermore, Homopteran pests which possess piercing-sucking mouthparts, that do not normally ingest insect pathogens applied to their host plants, can also be targeted (Dillon & Charnley 1991).

Entomopathogenic fungi have been identified in all divisions of the fungal kingdom. Interesting species are found within the Oomycetes, Chytridiomycetes and the Trichomycetes, but the most prevalent are located in the Deuteromycotina and Zygomycotina (Hall and Papierok 1982). In fact, more than 700 species of fungi from over 90 genera are known to be pathogenic to insects (Charnley 1989a). Ease of *in vitro* culture and broad host range, however, has led researchers to concentrate their interests predominantly on the deutermomycetes (Charnley 1989b). *Metarhizium anisopliae,* for example, has been identified from *ca.* 300 species of Lepidoptera, Coleoptera, Orthoptera and Hemiptera (Charnley 1991) although individual strains are selective (Zimmerman 1993). Although still very limited relative to their chemical counterparts, mycopesticides have and are experiencing commercial interest all over the world. Figure 1.2 lists some examples of currently registered mycoinsecticides.

Product	Fungus	Pest Insect	Producer	Comments
Mycotal	*Verticillium lecanii*	Whitefly & thrips	Koppert, Holland	
Vertalec	*Verticillium lecanii*	Aphids	Koppert, Holland	
BIO 1020	*Metarhizium anisopliae*	Vine weevil	Bayer, Germany	Registered, but not on sale, other products under development
Biogreen	*Metarhizium anisopliae*	Scarab larvae on pasture	Bio-care Technology, Australia	
Metaquino	*Metarhizium anisopliae*	Spittle bugs	Brazil	600,000 ha sugarcane treated annually Produced by cooperatives or cottage industries
Bio-Path	*Metarhizium anisopliae*	Cockroaches	EcoScience, USA	
Bio-Blast	*Metarhizium anisopliae*	Termites	EcoScience, USA	
Mycotrol GH	*Beauveria bassiana*	Grasshoppers, locusts	Mycotech, USA	
Mycotrol WP	*B. bassiana*	Whitefly, aphids, thrips	Mycotech, USA	
Naturalis-L	*B. bassiana*	Cotton pests, including bollworms	Troy Biosciences, USA	
Conidia	*B. bassiana*	Coffee berry borer	AgrEvo, Germany	
Betel	*B. bassiana*	Sugar cane white grub	NPP (Calliope) France	
Ostrinil	*B. bassiana*	Corn borer	NPP (Calliope) France	
Boverin	*B. bassiana*	Colorado beetle	Russia	Some commentators suggest quite large-scale use (>10000ha) but this has been disputed.
Boverol	*B. bassiana*	Colorado beetle	Czech Republic/Slovakia	
Boverosil	*B. bassiana*	Colorado beetle	Czech Republic\Slovakia	
-	*B. bassiana*	Colorado beetle	Poland	Name not known
-	*B. bassiana*	Various forest and crop pests	China	Produced by cooperatives or cottage industries Around 10 000 tons of conidial powder used to treat 0.8-1.3 million ha.
Engerlingspilz	*B. brongniartii*	Cockchafer	Andermatt, Switzerland	
PFR-97	*Paecilomyces fumosoroseus*	Whitefly	ECO-tek	Formerly owned by W.R.Grace
PreFeRal	*P. fumosoroseus*	Whitefly on glasshouse vegetables and ornamentals	Biobest, Belgium	Under licence from ECO-tek

Table 1.2 Commercial scale production of mycoinsecticides (From Charnley 1997)

A number of factors limit the competitiveness of mycoinsecticides against the undeniably effective and convenient chemical agents. Figure 1.3 summarises key areas for improvement. Those factors affecting the actual efficiency of infection will now be discussed.

1.	Widen host range within a pest complex but minimise non target effects (especially on parasites and predators of aphids)
2.	Improved tolerance of low humidity and improved infection at low humidity
3.	Improved growth and infection at different temperature extremes
4.	Faster *in situ* growth and hence faster kill at economical doses
5.	Improved sporulation on dead insects and thus improved disease spread
6.	Greater and broader fungicide tolerance.
7.	Improved inoculum shelf-life and easy storage
8.	Reduced cost by cheaper production (better productivity and infection)
9.	Greater understanding of processes involved in pathogenicity in order to optimise the above processes.

Table 1.3 Suggestions for areas of improvement for the use of fungi as pesticides (Quinlan 1988, Gillespie and Moorhouse, 1989, Milner 1997).

One of the principal limitations of using mycopesticides is that of their dependence on environmental factors for survival and virulence to the target pest.

Relative humidity (RH) is a critical factor since free water is an essential requirement for germination. Deuteromycete fungi generally require 92-93% RH for germination (Ferron 1981). Consistent with this, the spread of sporulation appears to occur during periods of high humidity or rainfall (Hall and Papierok 1982, Wraight and Roberts,

1987). Many fungi may still be able to infect insects at lower RH than this, since the surface of the plant and/or insect may provide a favourable microclimate enabling germination and thus infection (Hall and Papierok 1982). Given this dependence on humidity, the ability to germinate at low water potentials would be a desirable trait to include in a strain selection programme (Chandler et al. 1994, Matewele et al. 1994). Gillespie and Claydon (1989) suggested that current studies should be directed towards pests living in humid micro-climates or careful formulation development - recent work on *M.anisopliae* has shown an oil-based formulations to give good control of locusts even at low humidity (Bateman et al. 1993).

Temperature is also a limiting factor to successful control by fungi. Thermal preferences of fungi vary, e.g. 20-25°C for *Verticillium* and *Beauveria*, and 25 - 30°C for *Metarhizium anisopliae*. The life cycle of the host is dependent on the temperature, furthermore, accelerated development in response to high temperature, may reduce the chances of infection. Temperature and humidity are interdependent. In the glasshouse, humidity rises as the temperature falls at night. While the high RH supports germination, environmental temperature may be too low for fungal growth (Hall and Papierok 1982).

Other factors such as wind dispersal of dry conidia and effects of UV may also influence the likelihood of an epizootic occurring, (Ignoffo and Garcia, 1992). Biotic factors such as mobility of the host, feeding locations of the host, reproduction rates and density of the host population will also determine the spread of disease following initial application (Hall and Burges 1979).

Glasshouse and soil environments where high humidities and constant temperatures may prevail, with the added protection from solar radiation, are particularly suitable environments for pest control by fungi. Strains of *Verticillium* and *Aschersonia* have been extensively studied in glasshouse environments for years (Leathers et al. 1993). Control of pests in open field, crop and forest systems, however, presents a problem to insect pathogenic fungi. Environmental conditions are often unpredictable and even adverse. Furthermore there may be incompatibilities with chemical pesticides used there and a low damage threshold of the plants.

Due to their intrinsic sensitivity to environmental factors the infective propagules of fungi, the conidiospores and blastospores themselves, may have short lives in the field (Leathers et al. 1993). Thus in order to improve their efficacy in the field and further stabilise the product, formulation is important. Wetters, stickers, humectants, UV protectants and thixotropic agents can be included in a formulation , provided that they do not adversely affect the product. To improve persistence, emulsions and substrates can also be included (Lane et al. 1991). If environmental conditions can be accurately forecast, then the formulation can be adapted as necessary.

Perhaps the most important limitation of fungi, and one that has restricted commercial exploitation, is their slow rate of kill. Fungi may take as long as several weeks to infect and kill a whole population of pests (Leathers et al. 1993). To make efficient use of mycopesticides, an understanding of their biology is necessary so that they are used under optimum conditions for their mode of infection and a more rational

approach taken to strain selection and improvement (Payne 1988). Recent developments in molecular biology suggest the possibility of using direct genetic manipulation to enhance the performance of mycopesticides (Leathers et al. 1993). Genes that specify limiting virulence factors or host range determinants could be isolated and modified to produce enhanced organisms. Progress has been made to this end. Transformation systems have been developed for *Metarhizium* and *Beauveria* (Goettel et al. 1990; Daboussi et al. 1989) and a number of pathogenicity/virulence genes have been cloned and sequenced (see next section).

1.2.2. Determinants of pathogenicity and virulence

In order to review the options available for rational strain improvement programmes it is necessary to identify the determinants of pathogenicity and virulence, either for direct selection of these determinants or to identify genes for manipulation (Clarkson and Charnley 1996). Virulence is a quantitative assessment of a pathogen's ability to infect and kill a host. Virulence determinants may govern the speed and efficiency of kill of different fungal isolates. Pathogenicity is a qualitative term, hence a pathogenicity determinant is one that is essential for infection. Determinants of pathogenicity and virulence can be identified by three main approaches (Charnley 1989b): Firstly, by examining isolates of a species for correlations between particular traits and pathogenicity; secondly by determining the pathogenicity of mutants hyper- or hypo-productive for a particular trait (this may involve the use of reverse genetics to produce a nul-mutant) and finally, studying the biochemistry of the host-parasite interaction, making use of specific inhibitors.

The general outline of disease development of a Deuteromycete entomopathogen is shown in figure 1.4.

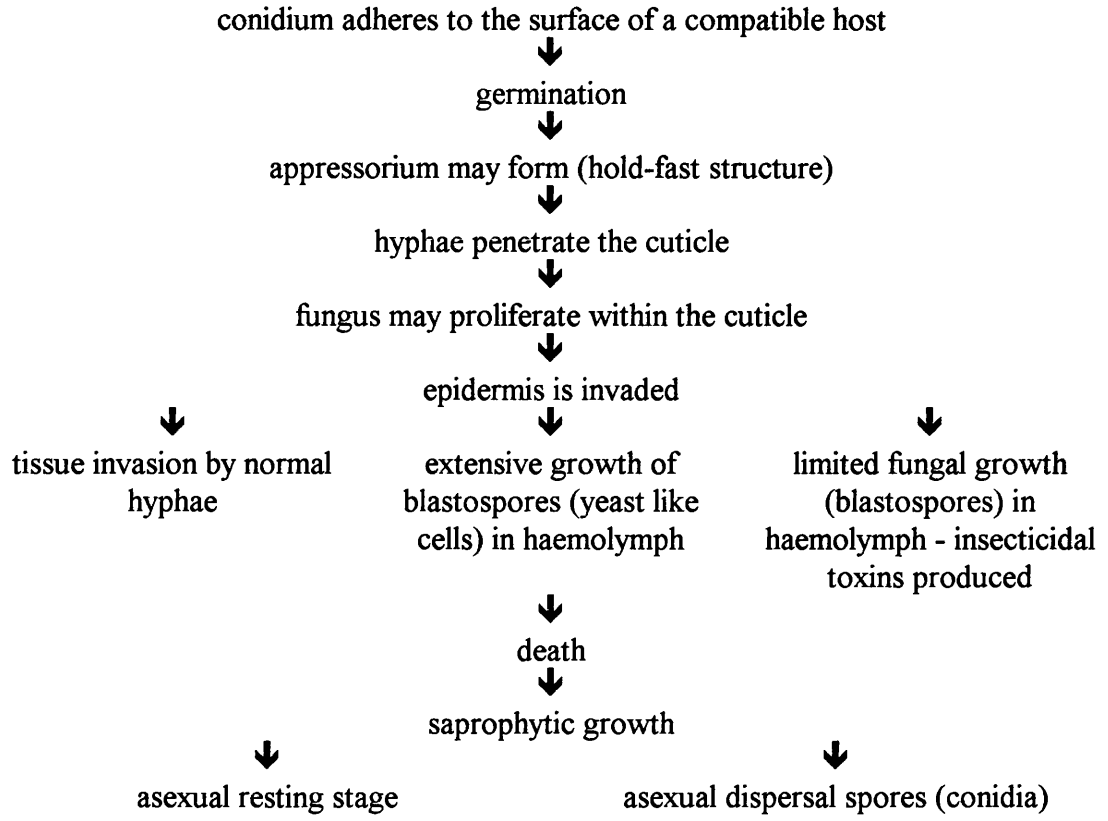


Fig 1.4. Diagrammatic representation of disease development of a Deuteromycete entomopathogen (taken from Charnley 1989b).

1.2.2.1 Adhesion of the spore to the cuticle

The success of entomopathogenic fungi is dependent on the ability of its infectious propagules to be delivered to a susceptible host and to be able to adhere to the surface of that host (Boucias & Pendland,1991).

The attachment of the fungal propagule to the host cuticle is mediated by both the chemical components on the outer layer of the spore wall and the epicuticle of the host. For fungi with terrestrial hosts attachment appears to be the result of specific

(e.g. glycoprotein, enzyme) and nonspecific (e.g. electrostatic or hydrophobic) recognition systems (Fargues 1984). Spores of *Beauveria bassiana, Metarhizium anisopliae* and *Nomuraea rileyi* are covered by layers of interwoven bundles of extremely hydrophobic rodlets that are important for adhesion (Boucias & Latgé 1988). In addition this rodlet layer may protect the spores from biotic and abiotic degradation (Boucias & Pendland, 1991). The protein hydrophobin is known to be an important component of the rodlet layer (St Leger et al. 1992a). Lectins have been detected on spore walls of these fungi, suggesting that these carbohydrate binding proteins could potentially also provide specific linkages, although hydrophobic forces appear to be the main controlling factor of adhesion for these fungi (Fargues 1984, Charnley 1989b). This initial attachment may be further reinforced by active secretion of adhesive materials or the modification of spore wall material/insect cuticle by conidial enzymes.

Fungi such as *V.lecanii, Hirsutella thompsonii* and some Entomophthorales use an amorphous mucus for spore attachment (Boucias & Pendland, 1991). This mucus coat may act as an anti-desiccant, to provide protection from toxic host polyphenols and an optimal environment for extracellular fungal enzymes in addition to acting as an adhesive. Many insect pathogenic fungi, such as *Nomuraea rileyi* and *M.anisopliae* actively secrete mucilage during germ tube and/or appressorial formation (reviewed by Boucias & Pendland, 1991).

The ability of spores to attach to an insect cuticle, essential for infection, may influence host specificity. Sitch and Jackson (1997) observed a greater deposition and retention of *V.lecanii* spores to host cuticles than non-host cuticles. In addition, hypovirulent strains of *M.anisopliae* failed to attach to the larval syphon of *Culex pipiens* (Al Aidroos & Roberts 1978). A knowledge of the

adhesion process is an important consideration for production and formulation technology, particularly with the current emphasis on formulating dry hydrophobic conidia as a wettable powder.

1.2.2.2. Spore Germination

Germination on the surface of the host is a prerequisite for infection and a number of studies have indicated a relationship between the speed of germination and virulence for example *V.lecanii* against the aphid *Macrosiphoniella sanborni* (Jackson et al. 1985).

Germination of *M.anisopliae* is initiated by water, however it is thought that progress to the first overt swelling stage is dependent on an exogenous carbon source (Dillon & Charnley 1990). A period of soaking in water prior to the addition of nutrients synchronised and accelerated swelling with germ tube and appressorial formation. Interestingly, the nutrients required for germination appear to relate to the specificity and host range of the fungus. Deuteromycete fungi with broad host ranges such as *M.anisopliae*, appear to have non specific requirements in terms of carbon and nitrogen sources for germination, although individual isolates may have specific requirements (St Leger et al. 1994a). Entomopathogens with more restricted host ranges such as *N.rileyi* primarily infecting lepidopteran pests appear to respond specifically to diacylglycerols and polar lipids (Boucias & Pendland, 1984). Significant amounts of cuticle degrading enzyme activity, particularly endoprotease, are present on the outer walls of conidia of *Metarhizium anisopliae* (St Leger et al. 1991a)

suggesting that hydrolysis of the cuticle prior to germination may contribute nutrients for the initiation of swelling.

Successful germination, in addition to the ability to utilise available nutrients, requires a tolerance of potentially toxic substances found on the host surface. Short chain fatty acids on the surface of the cuticle have antifungal properties. However, it has not yet been determined that they are present in inhibitory concentrations *in vivo* (reviewed by Charnley 1989b). St Leger et al. (1988a) suggests that greater antifungal activity may occur among longer chain saturated and unsaturated fatty acids. Recently, Gomez et al. (1997) indicated that aldehydes produced by the glands of the stink bug were fungistatic to *M.anisopliae.* Interpretation is complicated by the potential for antagonistic and synergistic interactions between chemicals on insect cuticles against fungal pathogens (Butt et al. 1995, Charnley, 1989b).

By reducing the exposure time on the surface of the host, there is less chance of conidial removal and death prior to penetration, and a greater portion of this inoculum can go on to enter the insect. Thus in addition to selecting faster germinating strains, any method of reducing this exposure time on the cuticle could increase the efficiency of a mycoinsecticide. Hassan et al. (1989a) have shown speed and synchrony of germination and subsequent appressorial formation to be limiting factors in pathogenesis. They found that by presoaking conidia of *M.anisopliae* prior to application, to synchronise germination, increased mortality of *Manduca sexta.*

1.2.2.3. Pre-penetration events

Prior to invasion of the host, fungi need to alter their growth strategy. A wide range of entomopathogenic fungi produce appressoria (apical swellings on germ tubes for attachment to the host) from which penetration pegs develop to breach the host cuticle. These appressoria attach firmly to the host via mucilage and provide a stable platform for penetration. Such fungi include strains of *M.anisopliae, B.bassiana* and *Paecilomyces farinosus* (St Leger et al. 1989a). Not all Deuteromycetes appear to require an appressorium for penetration, *Verticillium lecanii, Cordyceps militaris* and some strains of *B.bassiana*, for example, produce germ tubes that may penetrate the cuticle near the conidium or may grow considerably before doing so (Hall and Papierok 1982).

In vitro studies by St Leger et al.(1989a) have indicated that the differentiation of appressoria formation by isolate ME1 of *M.anisopliae* is stimulated by low molecular weight complex nitrogen compounds on a hard surface; simple, low molecular weight chemicals are repressive. Such thigmotrophic and chemical stimuli for the production of appressoria, appear to initiate translation primarily during the second round of nuclear division, because differentiation is blocked if DNA and RNA inhibitors are applied before this time. Some Hemipteran isolates of *M.anisopliae* produced appressoria under high nutrient conditions (St Leger et al. 1992b). Such hosts may be covered in nutrient rich honey dew, thus the ability to produce infective structures that are not catabolite repressed may be a specific adaptation to invade such insects.

The strategy for invasion appears to be controlled at the level of the cuticle. Butt et al. (1995) showed *M.anisopliae*, isolates V208, V245 and V248, to produce relatively few appressoria on aphid cuticle compared to the harder beetle cuticle. It would appear that the fungus penetrates more directly through soft bodied cuticles. Strains of *V.lecanii* able to penetrate directly the aphid *M.sanborni* appeared to be more virulent than strains exhibiting a delayed penetration, (Jackson et al. 1985). Extensive growth of fungi on hard cuticles may aid location of thinner or softer cuticle more easily penetrated by the fungus (Charnley 1984). Wraight et al. (1990) observed directional growth of *Erynia radicans* towards intersegmental cuticle of *Empoasca fabae*.

1.2.2.4. Penetration and the importance of cuticle degrading enzymes

The cuticle of insects, consists primarily of protein, chitin and lipid (shown in figure 1.5) It presents a formidable barrier to most potential pathogens. It is this barrier that entomopathogenic fungi must cross. Surface topography is important and some fungal pathogens such as *M.anisopliae* strain ME1 preferentially produce appressoria on flat surfaces, such as over hair sockets of early fifth instar larvae of *Manduca sexta* (St Leger et al. 1991a). The principle routes for fungi attempting to penetrate hard sclerotised cuticles appear to be arthrodial membranes (at joints and between segments) segmental cuticle and mouthparts, taking advantage, where possible, of wounds (Hall and Papierok, 1982).

Penetration may be facilitated by the action of citric and oxalic acids, produced by *B.bassiana* (Bidochka and Khachatourians 1991) and hydrogen peroxide produced by *M.anisopliae* against cuticular components, particularly proteins (St Leger et al.

1986a). The majority of entomopathogenic fungi, however, penetrate the cuticle by a combination of mechanical pressure and enzymic degradation (Charnley & St Leger 1991). Figure 1.6 is a diagrammatic representation of the process of penetration.

The epicuticle (outer layer) has a highly complex structure, lacking in chitin but containing phenol-stabilised protein. It is also covered by a waxy layer containing fatty acids, lipids and sterols (Andersen 1979). Its complexity makes it difficult to assign roles for particular fungal enzymes in the penetration of this layer. Furthermore, in many insects, it is apparently resistant to enzymic degradation. The fragile nature of the epicuticle, suggests that it may be more susceptible to mechanical pressure (St Leger 1995). Lipases and esterases have been located histochemically in and around penetrant structures on the epicuticle (St Leger et al. 1987a). However, the wax esters which are substrates for such enzymes are minor components of this layer compared to alkenes, alkanes and triacyglycerols. It is thought that lipoprotein lipases, together with chymoelastases, secreted by *M.anisopliae* and *B.bassiana* could aid the penetration of the inner epicuticle, which consists of polymerised lipoprotein (St Leger et al. 1987a).

The procuticle consists primarily of chitin fibrils embedded in a protein matrix together with lipids and quinones (Neville 1984). The proportions of chitin and protein, and the nature, extent of hydration, and cross linking of the proteins by quinones (sclerotization) will determine the mechanical properties of different cuticles and thus the mode of penetration. In soft cuticles, such as caterpillars, growth across the cuticle

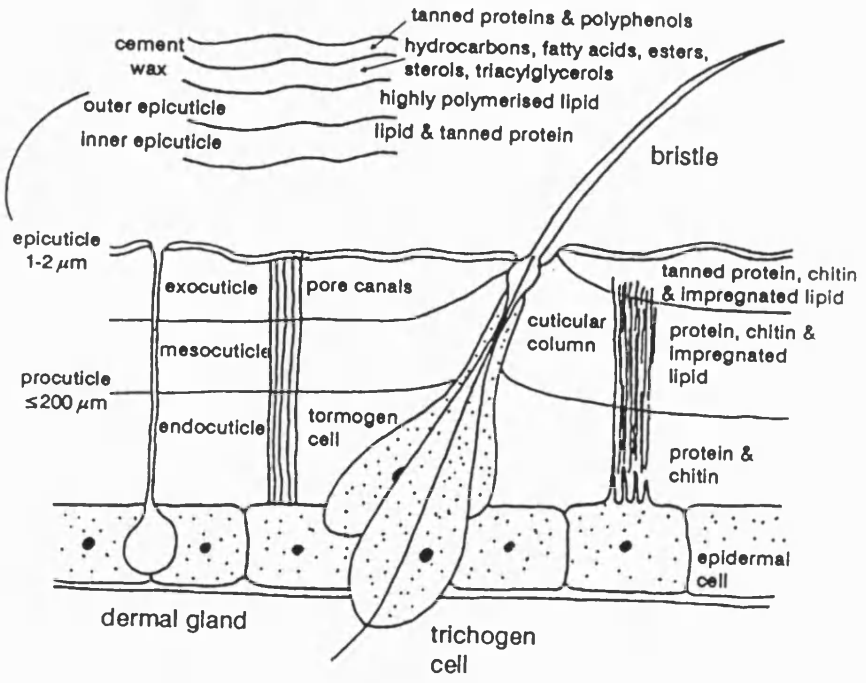


Fig. 1.5. Diagrammatic representation of a generalised insect cuticle

(Charnley 1989b)

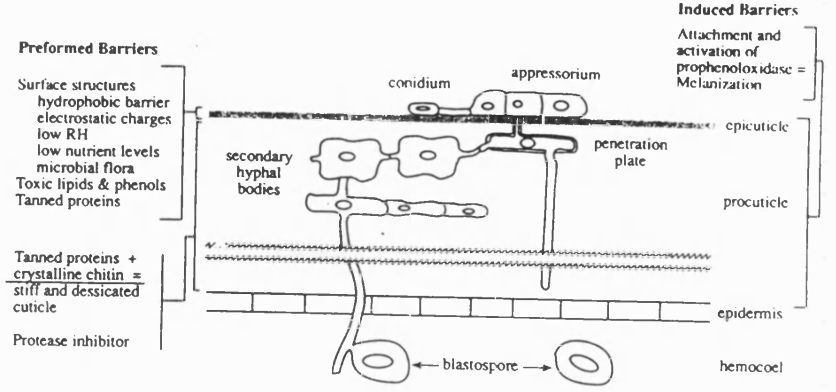


Fig.1.6. Simplified representation of penetration of the insect cuticle

by entomopathogenic fungi (Hajek & St Leger 1994)

is more or less direct. In hard cuticles such as wireworms, the fungus proceeds in a stepwise fashion (Charnley 1984). Lateral growth, within the subepicuticular region can cause fractures favouring penetration. The displacement of the lamellae by such penetrant hyphae is further evidence for the involvement of mechanical pressure in penetration (Charnley 1984). Enzymic digestion of protein in exocuticle might also occur to loosen cuticular lamellae, allowing an easier mechanical passage (Charnley 1989b).

The complex nature of the cuticle implies that the synergistic action of many different enzymes would be needed to degrade it. Several entomopathogenic fungi such as *Metarhizium anisopliae, Beauveria bassiana* and *Verticillium lecanii* have been found to produce a whole range of cuticle degrading enzymes when grown on cuticle *in vitro* (St Leger et al. 1986a,b). As found for *M.anisopliae*, esterase, endoprotease, aminopeptidase and carboxypeptidase appeared first, within 24h, followed by N-acetylglucosaminidase (GlcNAg-ase) which increased independently of chitinase. Only low levels of chitinase activity were found up to 3.5 days after inoculation, but this activity increased rapidly. Lipase, thought to be cell bound, was not detected until day 5 (St Leger et al.1986b). *Nomuraea rileyi*, differs from these fungi in that it produces high levels of protease and low levels of chitinase concurrently on host cuticle, indicating that chitinase may also be involved in early penetration events (El Sayed et al. 1993.) *N.rileyi* is a lepidopteran pathogen, and such larvae have more chitin in their cuticle than, for example, orthopteran insects (Neville, 1984).

The role of cuticle degrading enzymes is probably two fold. Firstly in facilitating penetration of the cuticle, and secondly in providing nutrients for the invading fungus. Mechanical pressure must predominate on sclerotised cuticles which are refractory to enzymic hydrolysis (St Leger et al. 1986a).

a. ***Chitinases***

Chitin constitutes 17-50% of the dry weight of the cuticle, and more pliant cuticles have a higher chitin content than stiff cuticles (Hillerton 1984).). Hassan & Charnley (1989a) found that when *M.anisopliae* is applied in conjunction with a chitin synthesis inhibitor, Dimilin, there was a synergistic effect against *M.sexta.* Later ultrastructural studies showed that penetration through Dimilin affected cuticle was greatly enhanced (Hassan and Charnley 1989b). Thus it would appear that in addition to being a stabiliser of the cuticular protein matrix, chitin also forms an important barrier to penetration.

Only very low levels of chitinase are produced during early stages of penetration of *Manduca sexta* by *M.anisopliae, M.flavoviride* and *B.bassiana*, although much greater levels are produced later in zones of proteolytic degradation (St Leger et al. 1996a). Chitinase is an inducible enzyme, and presumably the chitin in the cuticle is masked by protein and not available for hydrolysis until prior action by endoprotease (St Leger 1986c). Jackson et al. (1985) suggested a correlation between chitinase production and virulence for some strains of *V.lecanii* against *Macrosiphoniella sanborni* although a hypoproductive mutant was just as pathogenic to aphids as the wild type (Jackson et al. 1985). The majority of studies to date have centred around insects with

relatively thin unsclerotized cuticles, this may have limited our understanding of the role of chitinases in penetration. Insects with thicker cuticles or sclerotized cuticles would provide a more formidable physical barrier, which, by delaying penetration of hyphae, may allow sufficient time for chitinase induction and weakening of the chitinolytic barrier to infection (St Leger et al. 1991b).

Stirling et al. (1979) suggested that the end product of chitinase attack is chitobiose however preparations of chitinase from *Verticillium alboatrum,* for example, have been shown to release N-acetylglucosamine (GlcNAg)either as the sole reaction product or as a major end product along with di- and trisaccharides. GlcNAg was also found to be the major product (vs colloidal chitin) or only product (vs crystalline chitin) following chitin hydrolysis by *Metarhizium anisopliae* (St Leger et al. 1986a).

In contrast to chitinase, N-acetylglucosaminidase (GlcNAg-ase) is produced constitutively (St Leger et al.1986c) and could release monomeric inducers of chitinase from oligomers.

b. *Proteases*

Cuticular proteins appear to be the major barrier against hyphal penetration, however they are also the best nutrient source because chemically deproteinised grasshopper cuticle was a poor substrate for growth of *B.bassiana* (Bidochka et al. 1997a). Protein may account for up to 70% of the cuticle and a whole range of endoproteases have been characterised from entomopathogens including the collagenases produced by *Entomophthora coronata* (Zygomycetes) (Hurion 1979); subtilisin-like proteinases

(PR1), metalloproteases and trypsins produced by a range of Deuteromycetes (St Leger et al., 1987b, 1987c, 1994b) and chymotrypsins produced by *Erynia spp* (Zygomycetes) (Samuels et al.1990). Aminopeptidases and carboxypeptidases are amongst the secreted exopeptidases characterised from *M.anisopliae* (St Leger et al. 1993a, 1994c). The diversity of proteinase activities produced by entomopathogenic fungi highlights the importance of these enzymes not only for growth and survival of the organism, but also in adaptation to new environments and in pathogenicity. *M.anisopliae* is the most extensively studied fungal model of protease production to date (St Leger 1995).

For complete hydrolysis of cuticle proteins to amino acids to occur, the synergistic action of several proteolytic activities is required. Thus the proteolytic and peptidase enzyme systems of *M.anisopliae* complement each other in splitting most, if not all peptide bonds. For example the subtilisin PR1 has an active site which cannot readily accommodate proline. However, there is a dipeptidase that is specific for prolyl residues which overcomes the limitation of PR1 (St Leger et al. 1987c & 1993a). The interactions of the proteolytic complex of *M.anisopliae* during growth on cuticle are summarised in fig.1.7.

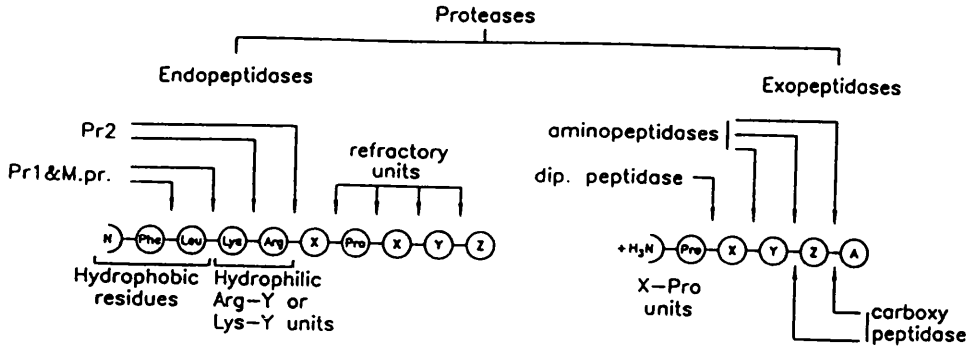


Fig. 1.7. The proteolytic complex produced by *M.anisopliae* during growth on insect cuticle. The proteolytic enzyme systems (subtilisin-like proteinases (PR1), metalloproteinase (M.pr.), trypsin-like proteinases (PR2), carboxypeptidase, aminopeptidases, and dipeptidyl (dip.) peptidase) complement each other in splitting most, if not all, types of peptide bonds. (St Leger 1995).

i) Subtilisins

Subtilisins are generally characterised as broad spectrum proteases, but they differ markedly in their activity on selected substrates, such differences may enable them to compete for different nutrients, and could influence host range (Segers et al. 1995).

PR1, a cuticle-degrading serine protease produced by *M.anisopliae* with activity versus chymotrypsin and elastase substrates, has been extensively studied (St Leger et al. 1987c, 1988b, 1989b). There are four isoenzymes of this enzyme with pIs of 10.2, 9.8, 9.3 and 9.0, (St Leger et al. 1994b). Two of these isoforms have been further studied, PR1a (pI ~ 10, M_r = 29kDa) and PR1b, (pI ~ 9, M_r = 31.5kDa) (St Leger et al. 1992c and Joshi et al. 1997 respectively). Despite only 53% homology of the primary amino acid sequences of their respective amino acid termini, these subtilisins appear to the exhibit the same physical and chemical properties.

Both enzymes appear equally effective at degrading a heterogeneous mix of insect cuticle proteins, suggesting a possible role in nonspecific degradation of protein during pathogenesis or saprotrophy (St Leger 1995). PR1a and PR1b possess a broad primary specificity for amino acids at P_1 with a hydrophobic side group at the second carbon atom (e.g. phenylalanine, leucine, methionine). PR1a differs from PR1b in that it prefers alanine over bulky hydrophobic groups at P_2 and P_3 (St Leger 1995).

PR1a is synthesised as a large precursor (40.3kDa) containing an 18 amino acid signal peptide, an 89 amino acid propeptide. The mature protein (28.6kDa) contains 281 amino acids (as revealed by characterisation of a cDNA clone, St Leger 1995). There is considerable sequence similarity with other enzymes of the subtilisin subclass of serine endoproteases and the serine, histidine and aspartate residues comprising the active site of these enzymes are conserved in PR1 (St Leger et al. 1992c).

V.lecanii, B.bassiana and *Aschersonia aleyrodis* have all been shown *in vitro* to produce analogous peptidases with alkaline, neutral or acidic pI points (St Leger et al. 1987b). Genes that are significantly similar to *pr1* are present in *Aspergillus flavus* and *V.lecanii*, and a *pr1*-like gene has also been cloned and sequenced from *B.bassiana* (Joshi et al. 1995). *Verticillium chlamydosporium,* an important pathogen of nematodes, produces a protease which is both functionally and serologically related to the PR1 from *Metarhizium anisopliae* (Segers et al. 1995).

Electrostatic binding of the positively charged PR1 to negatively charged groups on locust cuticle is a prerequisite to activity (St Leger et al.1986d). The basic nature of

PR1 allows it to bind to negatively charged cuticle groups, and it is thought that variations in the enzyme substrate binding would dictate the proteolytic efficacy against different cuticles (St Leger et al. 1986d). Acidic PR1 enzymes also attack locust cuticle, suggesting that charge differences over the surface of the cuticle allow both acid and basic proteases to bind to different regions of this cuticle (St Leger et al. 1986d). Following adsorption, the active site makes contact with susceptible peptide bonds and solubilised peptides are further degraded until a chain length of approximately five amino acids is obtained (St Leger et al. 1986d).

Biochemical evidence (reviewed by Charnley & St Leger 1991) suggests strongly that the extracellular chymoelastase, (PR1) produced by *M.anisopliae* is essential for cuticle penetration. A PR1-like enzyme is produced *in vitro* at high levels by all virulent isolates of Deuteromycete entomopathogens studied by St Leger et al. (1987b). PR1 has extensive cuticle degrading ability and has been shown to be produced *in situ* during infection (St Leger et al. 1987a, Goettal et al. 1989). Specific inhibition of PR1 using Turkey Egg White Inhibitor or a PR1 antibody, delayed *M.anisopliae* induced mortality of *Manduca sexta* by limiting fungal penetration of the insect cuticle. (St Leger et al. 1988b).

A PR1a nul- mutant produced by targeted gene disruption exhibited a partial loss of virulence of *M.anisopliae* to *Manduca sexta.* The onset of cuticle invasion by the mutant coincided with secretion of high levels of PR1b and metalloproteinase indicating that these other cuticle-degrading proteases substituted for PR1a (St Leger

1995). Thus, the full importance of proteases in pathogenicity may only be elucidated by the disruption of multiple genes.

ii) Trypsins

A 'classical' trypsin cleaves peptide bonds on the C-terminal side of basic amino acids and belongs to the serine protease group being inhibited by phenylmethylsulphonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP). *M.anisopliae* produces, *in vitro* and *in vivo*, a family of trypsin-like enzymes which have been named PR2 (St Leger et al. 1987a,c and St Leger et al. 1996b respectively). Although not as effective at degrading the cuticle as PR1, they have high activity against most solubilised cuticle proteins (St Leger 1995). They may be involved in the regulation and/or processing of PR1 (St Leger et al. 1988b). Furthermore, evidence to date suggests that PR2 isoforms complement digestion by PR1, by degrading the hydrophilic Arg-Y or Lys-Y units on the periphery of globular proteins (St Leger et al. 1996b).

The *M.anisopliae* derived PR2 occurs as multiple isozymes (~4.2-5.2) which have high activity versus solubilised cuticle proteins but not covalently bound (insoluble) cuticle proteins (St Leger et al. 1987c, 1994b) possibly due to poor adsorption to this substrate (Bidockha & Khachatourians 1994). The two major isoforms (pI 4.4, M_r = 30kDa and pI 4.9, M_r = 27kDa) share 56% homology between their N-terminal sequences, suggesting that they are related products of different genes. Sequence homology studies and analysis of cDNA clones have shown similarities to animal and bacterial trypsins (St Leger 1995) suggesting a strong selective pressure for the maintenance of trypsin specificity. A third trypsin-like enzyme with pI 5.0-5.5,

designated PR3, which possesses some cuticle degrading activity, has also been identified (St Leger et al. 1987c).

Analogous peptidases are produced by *V.lecanii, B.bassiana* and *A.aleyroidis.* Although these enzymes are specific for a Phe-Val-Arg group, in model substrates, they demonstrate less sensitivity to trypsin inhibitors than PR2 from *M.anisopliae* (St Leger et al. 1987b). In *Nomuraea rileyi* a trypsin-like enzyme is the major secreted protease (Gupta et al. 1993) suggesting that it has a role in non-specific protein degradation.

A cysteine protease, PR4, which exhibits trypsin-like substrate specificity, has also been characterised (Cole et al. 1993). Although cysteine proteases are widely distributed in nature, very little research has been conducted on those produced by microorganisms. PR4 has a pI of 4.6, a molecular weight of 26.7kDa and, although not as efficient as PR1, it has a higher cuticle degrading ability than PR2 (Cole et al. 1993).

Up to three isoforms of a metalloproteinase are produced by some strains of *M.anisopliae* and analogous enzymes are produced by *B.bassiana.* The metalloproteinase is a general proteinase showing activity to a wide range of proteins including insect cuticle and has the same substrate specificity as PR1 (St Leger et al. 1994b, St Leger 1995). The metalloproteinase is a general proteinase showing activity to a wide range of proteins including insect cuticle

M.anisopliae also produces three aspartyl (acidic) proteases pI 5.5-6.9 under acidic conditions (St Leger et al. 1998). This class of protease is not thought to be involved in cuticle degradation.

iii) Exoproteases

Exoproteases may also have an important role in the disease process. Aminopeptidase activity has been observed during penetration of blowfly wings by *M.anisopliae* (St Leger et al. 1987a) and may combine with PR1 to enhance release of amino acids from cuticle (St Leger et al.1991a, 1993a). The two main classes of aminopeptidase isolated from cuticle grown cultures of *M.anisopliae* were classified as broad spectrum aminopeptidase M with optimal activity for alanine, the most common amino acid found in many cuticles, and a post-proline dipeptdyl peptidase IV, which removes X-prolyl groups (St Leger et al. 1993a, 1995a).

Serine carboxypeptidases are widely distributed in fungi, probably functioning to cleave off amino acids needed for nutrition from external peptides. A carboxypeptidase, which is catabolite repressible, has been purified from cultures of *M.anisopliae* grown on cuticle (St Leger et al. 1994c). The enzyme has a neutral pH optima, small molecular mass (30kDa) and a high pI (9.8). It shows a broad specificity towards amino acids with hydrophobic side groups in a series of N-blocked dipeptides, substrates with phenylalanine being the most rapidly hydrolysed (St Leger et al. 1994c).

Production of cuticle degrading enzymes *per se* may not allow a fungus to successfully invade its host. The fungus needs to be able to regulate enzyme output in response to environmental and physiological cues, thus allowing it to conserve such enzymes in environments where they are not needed and produce them in quantity when they are. Studies on the regulatory mechanisms of entomopathogenic fungal enzymes may further our understanding of their role in pathogenesis and host specificity. This is discussed in chapter 3.

1.2.2.5. Growth inside the insect and host defence

Melanization, i.e. the deposition of oxidised phenols in the cuticle by host phenoloxidase (po) is the first obvious response to infection. However in insect cuticles it seems to be only effective against weak or slow growing pathogens (Charnley 1989b).

Growth inside the haemolymph of the insect occurs as yeast-like blastospores, hyphal bodies or protoplasts. Insects appear to display two active defence mechanisms if the pathogen successfully invades - cellular and humoral (reviewed in detail by Gillespie and Kanost 1997). Cellular defence reactions include haemocytic encapsulation or phagocytosis (Vey and Götz 1986).

Plasmatocytes are the main phagocytic cell type and are also involved in the encapsulation process. The cell membrane of *Galleria mellonella* plasmatocytes has been studied, and shown to possess B-1,3 glucan receptors giving them the ability to recognise fungal cells (Vilcinskas et al. 1997). Nodules are multicellular haemocytic

aggregates that can entrap an invading organism, these aggregates may adhere to tissues (Gillespie and Kanost 1997) Activation of the prophenoloxidase (ppo) system has been implicated in the non-self recognition (Charnley 1989b). β1,3 glucans in the fungal cell wall trigger the cascade responsible for ppo activation, po is sticky and thus attracts haemocytes to accumulate and form the capsule. Invading organisms can also be encapsulated by a melanin coat, and this can sometimes occur without the participation of haemocytes (Gillespie and Kanost 1997).

In the humoral, non-cellular, response certain defence proteins are induced or activated by the presence of pathogen-derived molecules. Bidochka et al. (1997b) found that although several proteins induced in *Manduca sexta* by bacteria, β-1-3 glucan and *M.anisopliae* cell walls were common, other proteins showed induction specific to fungal challenge. An inducible protease inhibitor was detected in the haemolymph of *Anticarsia gemmatalis* which inhibited growth of *Nomuraea rileyi* (Boucias and Pendland, 1987). The capacity of insects to release inhibitors against fungal proteases may be a key influence in their susceptibility against entomopathogenic fungi (Vilcinskas and Wedde, 1997).

Fungi must somehow evade or inactivate these host defences. Some Entomophthoralean fungi form protoplasts in the haemocoel. The absence of a cell membrane containing β1,3 glucans prevents host recognition. The cyclodepsipeptide toxins, destruxins (dtx) produced by *M.anisopliae* appear to interfere with haemocyte function and suppress ppo activation (Huxham et al. 1986; Vilcinskas et al. 1997).

Although these toxins may function in particular to disable the immune system, they are more generally cytotoxic to insect cells (reviewed by Hajek & St Leger 1994).

However, if death occurs after only limited growth in the host, with notable tissue damage, this suggests the involvement of toxic compounds (Zacharuk 1973). A cyclic peptide from *M.anisopliae* called 'Destruxin' has been extensively studied for its toxic effects on the host. Samuels et al. (1988a) showed a correlation between paralysis in mycosed insects, limited fungal growth in the haemocoel prior to death, short time to death, high virulence and high dtx production *in vivo* and *in vitro*. Destruxin injections also caused initial tetany followed by flaccid paralysis, symptoms of *M.anisopliae* infection (Samuels et al. 1988b). These findings indicate dtx as a virulence determinant for some strains of *M.anisopliae*.

Other toxins include the cyclic depsipeptides bassianolide (*V.lecanii* and *B.bassiana*) and beauvericin (*B.bassiana*) which have antimicrobial activity and may paralyse host cells (reviewed by Hajek & St Leger 1994).

1.2.2.6. Saprophytic Growth

After death of the insect the fungus takes over the cadaver and, if conditions are favourable, it will emerge through the body wall and sporulate on the host surface. *Verticillium lecanii* however has been seen to sporulate on live insects several days before death (Hall 1976). Generally, fungal strains able to kill only after extensive fungal invasion sporulate well on the cadaver given favourable conditions, however those killing their hosts rapidly, by the primary action of toxins, often fail to invade the

host and sporulate effectively, possibly due to competition from saprophytes (Hall and Papierok 1982).

It is also important for the propagules of the fungus to be able to survive under unfavourable conditions. Under these circumstances, many fungi are able to find protection within the soil (Hall & Papierok 1982) or show adaptations for survival. In response to unfavourable conditions, for example, some species of Zygomycetes produce resting spores (zygospores or azygospores), characterised by a thick cell wall and usually an oil globule, inside the cadaver (Hall and Papierok 1982).

1.2.2.7. Spore Dispersal

Spores from the surface of cadavers can spread to other potential hosts by a number of different routes. Generally dispersal is passive, making use of environmental factors such as wind and water, and location of a host is a random process (Garcia and Ignoffo 1977). Fungi like *Coelomomyces psorophorae,* however, produce motile zoospores which employ chemotaxis in the aquatic environment to locate their host (Ceranius & Soderhall, 1984 cited in Charnley 1989). The Entomophthoraceae can forcibly discharge their conidia (Gillespie and Moorhouse 1989).

1.2.3. *Verticllium lecanii* (Zimm) Viegas (Deuteromycotina: Moniliales)

Verticillium lecanii (Zimm) Viegas is a widespread entomopathogenic fungus known to parasitise all stages of development of many insect groups, Arachnida and even other fungi (Brady 1979). It was first described as an insect pathogen parasitising the

scale insect *Saissetia (Lecanium) coffeae* (Walker) by Nieter in 1861 (cited by Gillespie and Claydon 1989). Formerly known as *Cephalosporium lecanii* this fungus is primarily a pathogen of aphids, scales and whitefly (Hall 1981) however some isolates have been found from other orders such as Coleoptera, Lepidoptera (cited in Hall and Papierok 1982).

Verticillium lecanii can be found on infected insects forming cotton white/yellow colonies (Samson and Rombach 1985). It optimally grows at temperatures between 15 and 25°C and humidities of 85-90%RH in the greenhouse. The growth form of *V.lecanii* is very distinctive. Conidiophores arise from the aerial mycelium. The ellipsoidal conidia themselves are aggregated in an amorphous mucus at the end of these stalk like structures, which may enable attachment to the substrate (Tanada & Kaya 1993). *V.lecanii* conidia are thought to be dispersed from these mucilaginous heads by a water splash mechanism or contact with a moving object (Heale 1988).

V.lecanii was developed in England, initially, as an aphicide for use in glasshouses. In 1981, Tate and Lyle marketed an aphid derived strain of the fungus (1.72) under the name of Vertalec™, and it was initially successfully introduced to control aphids on chrysanthemums. The following year, a whitefly derived strain (19.79) whose formulation was known as Mycotal™, was introduced for glasshouse control of whitefly on cucumbers and tomatoes (Hall, 1981).

Prior to the release of these formulations, extensive glasshouse trials had shown excellent control of *Myzus persicae* on Chrysanthemum, (Hall and Burges, 1979).

Success of *V.lecanii* had also been recorded towards other aphids such as *Brachycaudus helichrysi, Aphis gossypii* and *A.fabae* (Burge 1988). In the first year of commercial production, control of aphids by Vertalec in integrated control programmes under glass was good. In 75% of cases it fulfilled the very high standards of control demanded on ornamental plants (Hall and Papierok, 1982).

Unfortunately, Vertalec and Mycotal were discontinued in 1986 due to their small market potential and erratic performance (Gillespie & Moorhouse, 1989). However, widespread problems with pesticide resistant aphids, whitefly and thrips in greenhouses in North Western Europe have encouraged the return of Mycotal™ and Vertalec™,. to the market. They are now produced by Koppert B.V. in the Netherlands.

The controlled environment and favourable temperatures of the large glasshouses of the Netherlands, where ornamentals are cultured on a large scale, as shown in Figure 1.8., provide optimum conditions for these entomopathogenic fungi to create epizootics.

A great deal of interest now focuses on optimising control of aphids and whitefly with *V.lecanii* in these glasshouses. Again, much success has been obtained on crops such as Chrysanthemum, particularly due to the humidity control obtained by regularly fogging the crop with water (Helyer et al. 1992).

Under suitable environmental conditions, *V.lecanii* infections can last the length of the crop season which is approximately 3 months on chrysanthemums with aphids and 3-4 months on cucumber against whiteflies (Hall, 1985). Given the nature of such

infections, Helyer et al. (1992) suggest that *V.lecanii* could not only work effectively as a curative agent by the creation of an epizootic agent spreading from infected insects but also as a preventative treatment with repeated applications with a nutrient source.

Fig. 1.8. At B.E. de Lier B.V. in the Netherlands, biological control has been used for 4 years. In 1996, *only* biological control was used. The photograph shows a glasshouse of Gerbera where, in conjunction with the application of other natural enemies, good control of whiteflies has been achieved with Mycotal. During January and February, Mycotal is sprayed three times every week and then at seven day intervals as a preventative and curative control agent.

Season carryover is not uncommon with *V.lecanii*, Figure 1.9. shows whitefly sporulating with *V.lecanii* in December, although Mycotal had not been sprayed since spring of that year.

In development of *Verticillium lecanii* as a biological control agent for use in Integrated Pest Management (IPM), a number of factors are important. Firstly, the design of the application procedure needs to be tailored towards the feeding behaviour of the pest.

Fig. 1.9. Photograph taken in December 1996 at B.E. de Lier B.V. showing Mycotal infection of whitefly on Gerbera, residual from the Spring application.

Hall and Burges (1979) demonstrated that aqueous sprays of *V.lecanii* spores could not control *Macrosiphoniella sanborni* and *Brachycaudus helichrysi* satisfactorily, since both species feed on the plant stem and it is difficult to deposit the spore suspension on the aphids and stem. In contrast *Myzus persicae*, feeding predominantly on the leaves of the plant was successfully and consistently controlled by *V.lecanii*, and a single spray could introduce an infection that controlled the aphids for the duration of the crop.

In order to achieve acceptable control of the target pest within an integrated pest management programme, compatibility with other biological control agents is an important consideration. For example, *Verticillium lecanii* is compatible with the hymenopteran whitefly parasite *Encarsia formosa* (Hall 1982). Compatibility of *V.lecanii* with fungicides used for the control of phytopathogenic diseases already

used on that crop also needs to be further researched to optimise the use of this pathogen (Hall 1975, Hall 1981)

One interesting aspect for consideration for improvement of mycological control methods is that of utilising mixtures of fungi in the glasshouse. The interaction of strains of *V.lecanii* has not been widely studied although mixtures of strains 19.79 and 1.72 have been used as part of a biological control programme for pests of chrysanthemum (Helyer et al. 1992). Different strains of the fungus may exhibit different attributes and virulence strategies that could complement one another if combined in a mixture. Heale (1988), for example, suggested using mixtures of fungal strains with overlapping temperature optima to control pests at a wide range of temperatures where no single strain was available. Identification of those characteristics responsible for virulence towards a specific host pest would enable rational selection of isolates for mixtures for improved control.

This project formed part of a research programme where the overall objective was to identify strains of *Verticillium lecanii* that have contrasting virulence strategies that could be combined in a mixture. Two aspects of the disease process which may interact with isolate virulence have been addressed in this programme. Firstly, whether the isolate kills the host indirectly through extensive growth or directly by the production of toxic metabolites. Secondly, the role of cuticle degrading enzymes in host penetration and invasion. The latter has been addressed in this study. One objective was to seek isolates of *V.lecanii* which have cuticle-degrading enzymes which are less subject to catabolite repression and thus could be produced in greater quantity during

host penetration and invasion. These isolates could be combined in a mixture with isolates that are optimal in other respects, for example, with one that is a high toxin producer.

Chandler et al. (1993), attempted to obtain synergism between strain 1.72 (from *Macrosiphoniella sanborni*) and strain 19.79 (from *Trialeurodes vaporariorum*). However, the mean pathogenicity of a mixture of the two strains on *Trialeurodes vaporariorum* and *Macrosiphoniella sanborni* was found to be intermediate to that of the individual strains, indicating a degree of competition, antagonism or incompatibility between the strains. Pathogenicity characteristics of strains on the same host vary greatly and it is the differential expression of these characteristics such as spore attachment, germination and suppression of host immune response that may lead to competition between strains. Interestingly only conidia of one strain were recovered from *T.vaporariorum* whereas conidia from both strains were recovered from *M.sanborni* (Chandler et al. 1993).

In the present study, the mode of regulation has been investigated of PR1-like proteases from 5 isolates of *V.lecanii*. I hoped to identify isolates that had contrasting but complementary enzyme regulation, e.g. early host specific induction and production in the haemolymph due to low impact of catabolite repression. Such isolates could then be combined in an attempt to produce a mycopesticide preparation with enhanced killing power.

2 Materials and Methods

All chemicals and enzyme substrates, unless otherwise indicated were purchased from Sigma chemical company Ltd. Media constituents were supplied by LabM and BDH. Products for isoelectric focusing were supplied by Pharmacia Biotech and BioRad.

2.1 Growth conditions

2.1.1 Culturing the fungus

Five isolates of *Verticillium lecanii*: **Vertalec (KV71), Mycotal (KV01), KV22, KV54** and **KV42**, provided by Koppert B.V., the Netherlands, were investigated for the production of PR1 and PR2-like enzymes. These isolates were cultured routinely on malt agar (2% w/v malt extract, 2% agar) and malt agar supplemented with bacteriological peptone (5%) at 23°C . Conidial suspensions were prepared from actively growing cultures (10 days old) by washing with sterile dH_20 and filtering through 2 layers of sterile muslin.

For strain comparison experiments, liquid cultures were grown in modified basal salts plus trace elements (Cooper and Wood 1975), which contained per litre 1g KH_2PO_4, 0.5g $MgSO_4.7H_20$ and trace elements at ppm: 0.2 $FeS0_4.7H_20$, 1.0 $ZnS0_4.7H_20$, 0.02 $NaMo0_4.2H_20$, 0.02 $CuS0_4.5H_20$ and 0.02 $MnCl_2.4H_20$, plus 50mM 2-[N-morpholino] ethanesulphonic acid (MES - a non-metabolizable buffer) and buffered to pH6. Ground locust cuticle was supplied at 1% (w/v).

For transfer experiments, liquid cultures were grown in complete medium, which consisted of Modified Czapek Dox (Oxoid) supplemented with 2 g l^{-1} each of casein hydrolysate, mycological peptone, yeast extract and malt extract.

Carbon/nitrogen sources, were supplied at 1% (w/v), since BSA concentrations above 1% have been found to repress protease production (Paterson *et al* 1994a).

2.1.2 Preparation of Ground Insect Cuticle

Cuticle from adult desert locusts (*Schistocerca gregaria)* was prepared according to the method described by Andersen (1980). Approximately 100 locusts were frozen at -20°C for 1 hour and then homogenised in a Waring blender in 1% w/v potassium tetraborate. The cuticle pieces were washed extensively in distilled water, stirred overnight in 1% (w/v) potassium tetraborate and then air dried at room temperature. The cuticle pieces were then milled to a fine powder using a coffee grinder. The powder was washed in 1% potassium tetraborate and finally in distilled water, allowed to settle and any floating material removed. Aphid cuticle from *Myzus persicae* was prepared similarly.

2.1.3 Preparation of de-proteinised chitin

Practical grade chitin from crab shells was hydrolysed in 30% (w/v) potassium hydroxide (KOH) at 80°C for 2 hours to remove all trace protein. The chitin was removed from the hydrolysate, washed extensively in distilled water and then allowed to air dry.

2.2 Strain Profiles of Enzyme Production on Cuticle

100ml of culture medium containing basal salts and 1% (w/v) of ground locust cuticle was inoculated with 1 x 10^7 conidia and incubated at 23°C in an orbital shaker (120 rpm.). 3 replicate flasks for each of the five strains were incubated under these conditions, and 1ml aliquots from each of the cultures removed daily for eight days. The aliquots were centrifuged at 11600g for 3 minutes, to separate the fungal material and undigested cuticle from the supernatant. Each supernatant was tested for its activity against the two substrates for PR1 and PR2-like activity (section 2.4)

2.3 Transfer Experiments.

Complete medium, 100ml in 250ml conical flasks, was inoculated with 4 x 10^6 conidia and incubated at 23°C, in an orbital shaker (120 rpm.) for 4 days to establish extensive fungal growth. (Morphology of fungal strains is discussed in chapter 3, section 1). The culture was sieved through 2 layers of sterile muslin, and the retained mycelium was washed with sterile basal salts medium, transferred to 100ml basal salts medium and starved for 24 hours under the same conditions as above to ensure complete catabolite de-repression. The mycelium was then transferred to different carbon substrate conditions at 1% (w/v) and the culture supernatant was assayed for PR1-like activity at 16 and 36 hours.

For carbon and nitrogen de/repression experiments, the fungal biomass was transferred to basal salts deficient in one or both of C or N plus cuticle. The medium used was

buffered basal salts plus trace elements plus sucrose (1%w/v) or NH_4Cl (0.4% w/v). All transfer experiments were replicated 4 times for each strain of *V.lecanii* tested.

2.4 Enzyme assays.

PR1-like activity was assayed by monitoring the release of nitroanilide (NA) at 405nm from the peptide substrate Suc-Ala-Ala-Pro-Phe-NA as described by St Leger et al (1987a). PR2-like activity was assayed by monitoring the release of NA from the substrate Bz-Phe-Val-Arg-NA. The reaction volumes used were 200µl 0.225M Tris/HCl buffer, pH8; 40µl of sample to be tested and 50µl of 2mM solution of substrate in Dimethyl Sulphoxide (DMSO). The actual sample was diluted accordingly to ensure a linear reaction over 3 minutes. The release of nitroalanine was monitored at 405nm using a Dynatech MR5000 microtitre plate reader. Activity was expressed as nkat NA released ml^{-1} min^{-1}, as calculated from a standard curve of nitroalanine standards. 1 unit is defined as the amount of enzyme required to convert 1µmole of substrate per minute. 1 unit of enzyme is equivalent to 16.67 nKats (Katals).

2.5 The use of ergosterol as a measure of fungal biomass

Determination of biomass in fungal cultures grown on an insoluble nutrient source has to be done indirectly. Ergosterol is a fungal sterol found in few organisms other than higher fungi (Martin et al, 1990) and Janet Graystone, at Bath, has shown a strong correlation between yield of ergosterol and fungal mass for Vertalec. Therefore ergosterol content can be used to determine fungal biomass by reference to a calibration graph.

2.5.1 Ergosterol Extraction

The procedure used for the extraction and quantification of ergosterol was a modification of the method described by Seitz et al (1979). Exactly the same amount of *V.lecanii* (Vertalec), as measured into sterile dishes on a balance, was transferred into the different substrate conditions as per the transfer experiments. 24 hours post transfer, the biomass of fungus was extracted from each liquid culture by sieving through two layers of sterile muslin and washing with distilled water. The retained material was ground to a fine powder in liquid nitrogen and then lyophilised for 48 hours or until all the water had been removed.

The fungal material was then transferred to a round bottomed flask containing 20 ml of Analar ethanol. The experimental solution was covered with blackout material to prevent breakdown of ergosterol into ergocalciferol (vitamin D) (Tuksida, 1980). 2ml potassium hydroxide (KOH), pyrogallol (an antioxidant) and anti-bumping granules were added to the mixture which was then refluxed for 30 minutes.

Note: all glassware used in the sterol extraction was washed in Decon, rinsed twice in distilled water, acid washed and rinsed again in distilled water prior to use.

2.5.2 Ether extraction of the non saponifyable fraction

The sample was washed with diethyl ether three times in a separating funnel and then washed with water to remove all the ethanol. The ether extract was then rotary evaporated at 40°C, any residual water was then removed by lyophilisation.

2.5.3 High Performance Liquid Chromatography (HPLC)

To prepare the sample for HPLC analysis, it was re-dissolved in 2.5ml methanol and passed through a filter to remove any particulate matter.

HPLC was performed in a Gilson system comprising two 303 pumps, an 811b dynamic mixer, a 401 dilutor, a 231 sample injector, a Rheodyne injection valve fitted with a 100µl loop and a Gilson 118 UV dual wavelength detector. The system was controlled by the Gilson 714 manager on an Viglen PC.

Ergosterol was separated and quantified by reverse phase HPLC, using Spherisorb C18 column with detection set at 282nm. Ergosterol has a pair of conjugated double bonds at carbons 5 +6 and 7 + 8 giving it a different UV absorption spectrum to other sterols, thus measuring UV absorption at 282nm enables it to be detected against a background of other sterols which usually absorb very little light at wavelengths above 282nm.

A mobile phase of 95% methanol and a flow rate of 1ml a minute gave good separation of the ergosterol peak from other extracted sterols. An ergosterol standard (Aldrich) gave a retention time of around 35 minutes. Results were compared to the Vertalec calibration curve, for 4 day old cultures, developed by J Graystone at Bath.

2.6 Estimation of pH optima of PR1-like enzymes from within culture filtrates from transfer experiments

To further substantiate the results from the induction experiments, by ensuring that enzyme activity was optimal at pH8.0 for all conditions, filtrates from cultures of Mycotal and Vertalec were assayed for PR1-like activity at a range of pH conditions using a series of overlapping buffers (figure 2.1). The concentration of each buffer was that of Tris-HCl used in all other assays (0.225M).

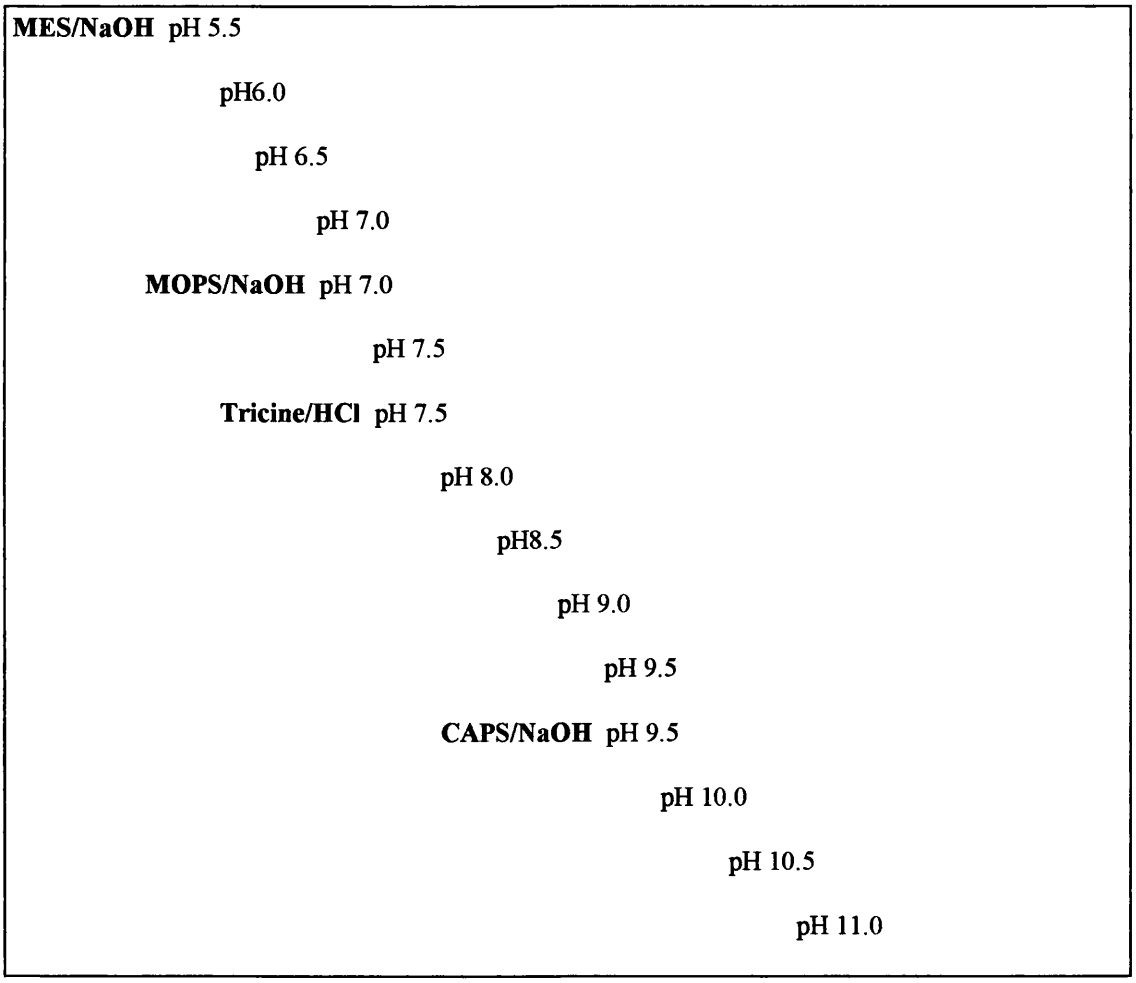


Figure 2.1. pH values and buffers used to test pH optima of PR1-like enzymes

MES = 2, - [N-morpholino] ethanesulphonic acid
MOPS = 3,-[N-morpholino] propanesulphonic acid
Tricine = N-Tris[hydroxymethyl] methylglycine
CAPS = 3-[cyclohexylamino]-1-propanesulphonic acid.

2.7 The effect of N-acetyl glucosamine (GlcNAg), when supplied in a restricted manner on the production of PR1-like enzymes by lead strains of *Verticillium lecanii*

2.7.1. Establishment of constant rates of diffusion of GlcNAg from diffusion capsules

N-acetylglucosamine was supplied to cultures at linear flow rates in a restricted manner by means of diffusion capsules (Pirt, 1971). Rates were controlled by altering the number of membranes (dialysis tubing) through which diffusion occurred and the concentration of GlcNAg within the capsule. Empty diffusion capsules were washed in absolute ethanol and then autoclaved with 5 or 10% (w/v) GlcNAg at 115°C, 0.1MPA pressure for 10 minutes before placing in 250ml conical flasks containing 100ml basal salts. The release of GlcNAg was monitored over time using the Nelson-Somoygi Reducing Sugar Assay (2.7.2).

Once near linear rates of flow of the monomer had been established, capsules were supplied to starved cultures of *V.lecanii* to find an appropriate release rate that could be utilised by the fungus, without excessive build-up within the medium over the period tested. Conditions tested were 5%, 10% GlcNAg with 1, 2 or 3 membranes covering the aperture.

2.7.2. Nelson-Somoygi assay for N-acetyl glucosamine

The Nelson-Somoygi assay (Nelson, 1944). was used to detect any changes in reducing sugar present, and thus to indicate changes in the concentration of GlcNAg

present. The composition of Nelson-Somoygi solutions is described in Appendix 1. 500µl of stopping reagent (reagents A & B) was added to 1ml of the sample to be tested. The reaction mixture was incubated at 95°C for 1 h and allowed to cool before adding 500µl of Nelson C. 200µl of each sample were then dispensed into individual wells of a microtitre plate and the absorbance at 595nm read on a Dynatech MR5000 plate reader. A standard curve of GlcNAg was similarly prepared.

2.7.3 The effect of GlcNAg on PR1-like activity

An established biomass of Vertalec or Mycotal was starved for 24 hours and transferred to buffered basal salts supplemented with a diffusion capsule containing GlcNAg or an appropriate control (full details are given in 3.3.5). 4 replicate cultures were incubated at 23°C, 120rpm. Samples were removed at 16 hours and fungal matter was removed by centrifugation as described previously. PR1-like activity was measured by monitoring the rate of release of nitroalanine from the substrate N-Suc-Ala-Ala-Pro-Phe-pNA.

2.8 Comparison of host and non-host cuticle on PR1-like production by lead strains of *V.lecanii*

Cuticle from *Myzus persicae* was extracted as for locust cuticle, by the method of Andersen (1980). Aphids were collected over a period of time from stock cages on pepper and through a generous gift from Robert Lind (Zeneca Agrochemicals). 4 day old 'mini' cultures of Vertalec, KV42 and Mycotal were established in 25ml flasks

containing 10ml complete medium. Following starvation, they were transferred to flasks containing buffered basal salts supplemented with 0.1g aphid or locust cuticle. PR1-like activity was monitored at 16 and 36 hours.

2.9 Flat-bed isoelectric focusing

Culture filtrates 24 h post transfer (unless otherwise stated) into the test conditions as described were dialysed against several changes of 1% glycine for 24 h, followed by dH_20 for a further 24 h to eliminate salt from the samples which could interfere with isoelectric focusing. Samples of filtrate to be analysed by isoelectric focusing were concentrated by lyophilisation. 50µg of protein was loaded into each lane of a broad range Ampholine PAGplate (Pharmacia Biotech) - a precast IEF gel, pH 3-10, by means of sample application pieces placed 3 cm from the cathode. Samples were then focused for 1.5h at 10°C at a constant voltage of 30W. Gels were then either developed with Coomassie Blue R-250, qualitatively assayed for protease activity using gelatin or gel slices were excised and tested for chymotrypsin activity. A broad pI calibration kit was used to estimate pIs of proteins present.

Protein concentrations in samples were estimated by measuring the absorbance at 260 and 280nm of the sample, using a Cecil 2040 spectrophotometer, and inserting these values into the following equation.

$$[1.55 \times A_{280}] - [0.76 \times A_{260}] = \text{amount of protein/ mg protein ml}^{-1}$$

2.9.1 Coomassie Stain

Immediately after isoelectric focusing, the gel was placed in 250ml fixing solution (29g Trichloroacetic acid and 8.5g sulphosalicylic acid in 250ml dH_20) for 1 hour, to precipitate proteins and allow the carrier ampholytes to diffuse out of the gel. The gel was washed once in destaining solution (500ml ethanol, 160ml acetic acid in 2l dH_20) for 5 minutes and then stained in Coomassie staining solution (preheated to 60°C for 10 minutes. The gel was destained using several changes of destain until the background was clear. Gels were then soaked in preserving solution for 1 hour and then placed on a glass plate with the gel side up, covered with cellophane soaked in the preserving solution. The gel was allowed to dry at room temperature.

2.9.2 Identification of proteases - use of Gelatin overlay

Bands on the IEF gels were characterised as being proteases according to their ability to degrade gelatin. Photographic film, was soaked in distilled water for 15 minutes prior to use. Immediately after focusing, gels to be tested, were overlaid with pieces of film, cut to the shape of the gel. Any proteases present were shown to degrade the gelatin coat of the film. The overlay was monitored at regular intervals, noting the position of bands as soon as they first became apparent. The film was left in contact with the gel until no further bands of degradation were observable. Over the course of the experiment, the film was photographed using a UVP whit transilluminator using a 'Grab-It' Annotating Grabber 2.04.5.

2.9.3 Gel slice assays

Using a mounted needle, scalpel and grid, 1mm slices were excised from the gel from the direction of the cathode for each sample lane. These slices were transferred to wells of a microtitre plate containing 100µl Tris-HCl (pH8.0) buffer and homogenised using a multiple well homogeniser, then 50µl N-Suc-Ala-Ala-Pro-Phe-pNA or Bz-Phe-Val-Arg-pNA was then added and the release of nitroalanine monitored over 1 hour.

2.9.4 The effect of specific inhibitors on enzyme activity

Gel slices homogenised in Tris-HCl buffer were then incubated with the protease inhibitors Chymostatin (Phe-(Cap)-Leu-Phe-al); Leupeptin (N-acetyl-leu-leu-arg-al) or Turkey Egg White Trypsin Inhibitor at concentrations of 100µM, 100µM and 0.1% respectively for 1 hour. Distilled water or Dimethylsulphoxide (DMSO) were used as negative controls in each case. Enzyme activity was then measured as in section 2.9.3 and expressed as a percentage of activity without the inhibitor.

2.10 Cuticle Digestion Assays

Samples of each PR1-like isozyme produced by three of the strains of *V.lecanii* tested (Vertalec, KV42 and Mycotal) were eluted from focused IEF gels and concentrated as described in section 2.12. Enzyme and protein concentrations were measured for each

form. A comparison of each isozyme's ability to degrade aphid or locust cuticle was made.

5 mg of cuticle was transferred into each 1.5ml eppendorf tube and washed in several changes of 1ml 2mM Tris-HCl (pH 8.0) over 3 hours. The buffer was then replaced by 950µl 10mM Tris-HCl buffer (pH8.0) supplemented with 50µl PR1-like enzyme to be tested. PR1-like enzymes were also incubated with Turkey Egg White inhibitor (1mg ml^{-1}) for 20 minutes on ice before adding to the cuticle. Suitable controls, i.e. cuticle and buffer alone, buffer alone, and buffer plus TEW inhibitor were included. Tubes were then incubated at 28°C overnight on a cyclogyrator. After incubation, samples were centrifuged at 11600g for 3 minutes before assaying for protein concentration using a UV spectrophotometer (section 2.9). The amount of protein released from the cuticle was estimated by comparison to that found in the above controls.

2.11 SDS-PAGE (Sodium Dodecyl Polyacrylamide Gel Electrophoresis)

Samples for analysis by SDS-PAGE were dissolved in loading buffer (pH6.8) and boiled for 3 minutes (10 minutes for antibody raising, section 2.13) immediately prior to loading on the gel (unless otherwise stated, 12.5% resolving gels were used). Gel electrophoresis was carried out at 150V for approximately 1 hour in Mini-PROTEAN II units (BioRad) according to Laemmli (1970). Gels were stained with 0.5% Coomassie blue R-250 in 50% methanol and 10% glacial acetic acid for 15 minutes. Destaining was performed in several changes of 45% methanol and 9% acetic acid.

The molecular weight of the protein was determined by measuring its mobility relative to broad range molecular weight markers (BioRad).

2.12 Protein Purification

One litre of filtrate was harvested from cultures of *V.lecanii* on locust cuticle, by filtering through 2 layers of muslin, and then Whatman filter paper in a Buchner Funnel. An overnight total protein precipitation was performed using 70% (w/v) Ammonium Sulphate, which was added slowly over a number of hours at 4°C. The filtrate was kept stirring at all times. The resultant suspension was centrifuged at 16270g for 1 hour. The pellet was re-suspended in 10% of its original volume.

The protein concentrate was then transferred to dialysis tubing, and dialysed for 24 hours against three changes of distilled water and 1 change of 1% glycine to remove all salt and ammonium sulphate. To obtain semi-purified forms of all isoforms present, this salt free protein sample was then concentrated further by dialysis against Polyethylene glycol (PEG, 20,000 MWT) for flat bed iso-electric focusing. To improve yields of the most basic protease (for raising of antibodies) the protein sample was not concentrated further and was used directly in preparative iso-electric focusing using the Rotofor®(Bio-Rad).

Preparative iso-electric focusing was carried out using a Rotofor cell (Bio-Rad). 2.5ml Bio-Lyte (Bio-Rad) ampholytes (pH range 3-10, 40%w/v) at a final concentration of 2.0% were added to the enzyme sample and the total volume was brought up to 50ml

with de-ionised water, and applied to the Rotofor®. The sample was focused for up to 4 hours at a constant wattage of 12 W until a constant voltage was obtained. Twenty fractions were collected and their pH measured immediately after collection. Ampholytes were removed from the fractions by dialysing against excess dH_20 for 24 hours. Fractions were then assayed for activity against the PR1 substrate, Suc-Ala-Ala-Pro-Phe-NA and for protein concentration. Positive fractions were then pooled and concentrated further, using PEG, for flat bed iso-electric focusing.

Samples were focused using the flat bed system as described in section 2.9, however one continuous sample strip, 3cm from the cathode, was used. Up to 8mg protein was loaded onto one pre-made gel (4mg for half a gel). After focusing, 1mm strips were cut from the gel with plastic backing attached, using scissors at the approximate pI of the isoforms to be tested (or just at the cathode if only the most basic form was required). The protein in each strip was eluted using a Tris-Glycine buffer (as that for SDS-PAGE without SDS) for 1 hour at 100V. Following elution, the dialysis tubing was further rinsed with dH_20 and the gel slice separated from the plastic backing and washed overnight. The washings were then pooled and the sample concentrated firstly using PEG and then by means of a 10kDa Micron® micro-concentrator (Amicon), ensuring that all particulate material had been removed by filtration. Levels of purity were assessed by means of SDS-PAGE and staining with Coomassie Blue. Samples obtained were then used in either cuticle digestion experiments or for raising and titreing of antibodies.

2.13 Antibody Production

2.13.1 Raising Antibodies

The most basic protease (pI ≥9.5) produced by each of Vertalec and KV42 was chosen as the form of PR1-like enzyme to raise antibodies to. For Vertalec, it could be purified to a single band and for KV42 it could be purified to a band easily excised from a PAGE gel without any interfering other proteins (as indicated by Coomassie and Silver Staining). 50µg of purified sample was dissolved in loading buffer and boiled for 10 minutes to ensure complete denaturation, before loading onto a SDS-PAGE gel. After the gel had been run to completion, it was washed in distilled water and then stained with Coomassie Blue dissolved in distilled water for ten minutes. After washing repeatedly with water, bands could be discerned as clear zones against a background of blue. The band of interest, as confirmed using broad range molecular weight markers, was excised and washed in several changes of dH_20. After lyophilisation it was ground, using an eppendorf homogeniser in 800µl sterile Milli-Q water. The suspension was then mixed with 500µl Freunds Complete Adjuvant (DifCo). The sample was then given to a licensed animal house (University of Bath) and used to inject a long eared rabbit. For subsequent boosts, Freunds Incomplete Adjuvant was used. Between 30 and 60µg protein was used to boost the rabbits every 14 days. Two rabbits were used for each strain. Test samples of blood were collected regularly and allowed to clot at room temperature for up to 4 hours in glass universal tubes. The tubes were then transferred to 4°C overnight, to allow the clot to detach from the wall of the vessel. The supernatant was then centrifuged at 17210g for 20 minutes. The serum was then stored with 0.05% sodium azide in aliquots at -20°C.

The level of antibody in the rabbit sera was tested regularly (as described below), and once no significant increase in antibody response was observed, boosting was stopped and the rabbits were exanguinated and blood collected.

2.13.2 Estimating antibody titre

In order to test for antibody production, a 'dot blot' method was employed, whereby the sample was applied directly onto nitrocellulose. 2µl of sample to be tested was applied onto a nitrocellulose membrane. The sample was baked onto the filter in an oven at 60°C. Once completely dry, the membrane was floated on Tris-Buffered Saline supplemented with Tween (TBST - 0.01M Tris-HCl, pH 8.0, 8.8g 0.05% Tween-20 in 1l dH_20). Once evenly wet it was rinsed again in TBST. Non-specific binding sites were saturated by blocking the membrane with 2% skimmed milk (Marvel) in TBST for at least one hour at room temperature. The nitrocellulose membrane was then washed for 20 minutes in four changes of TBST. Dilutions of each test serum were made (1:100, 1:500 and 1:1000) in TBS (TBST without Tween-20) supplemented with 0.2% skimmed milk. Membranes were covered in solution of antibody and incubated for 1 hour at room temperature with gentle shaking. Membranes were then washed in four changes of TBST over 20 minutes before transfer into the secondary antibody linked to horseradish peroxidase (1:1000 dilution in TBS plus 0.2% milk). Membranes were incubated at room temperature for 1 hour and then washed, as before in 4 changes of TBST. Developer was made fresh just before using and added straight to the dot blot, 9ml TBS, 1ml chloro-1-napthol (from a stock of 3mg ml^{-1}) and 10µl H_2O_2 (30%). If colour development was not observed

after 10 minutes, a further 10µl H_2O_2 was added. Membranes were allowed to dry and stored in foil, protected from light.

For increased sensitivity in dot blot assays, the Vectastain® ABC kit (Vector Laboratories) was used, which utilises the high affinity of Avidin, a 68000 MWT glycoprotein for the small molecular weight vitamin biotin. In addition, this is an irreversible reaction and the Biotin/Avidin system can be effectively exploited because avidin has 4 binding sites for biotin and most proteins (including antibodies and enzymes), thus macromolecular complexes between avidin and biotin can be formed. The ABC kit stages are unlabeled primary antibody, followed by biotinylated secondary antibody and a pre-formed avidin and biotinylated horseradish peroxidase macromolecular complex (ABC reagent). With this increased sensitivity, dilutions of 1:1000 to 1:4000 of 1° antibody could be used. The full protocol is given in section 2.1.3.3.

2.13.3 Western Blotting

Samples for testing were loaded onto a 12.5% SDS-PAGE gel. Once the sample buffer had run to the end of the gel, the gel was removed from the mount and rinsed in distilled water. It was then equilibrated at 4°C in transfer buffer (10mM CAPS, 3mM DTT and 10% MeOH, pH11) for 30 minutes as was two pieces of blotting paper and transfer pads and a piece of nitrocellulose cut to the size of the gel. A gel/membrane/blotting paper sandwich was prepared ensuring exclusion of all air bubbles. Electroblotting was then conducted at 4°C at 300mA for 1 hour. Once

electroblotting was complete, the membrane was then developed using the Vectastain ABC kit.

The membrane was immersed in 0.1% (v/v) Tween 20 in Tris-buffered saline (100mM Tris, 0.9% sodium chloride pH7.5, TTBS) for 30 minutes with gentle agitation. The membrane was then transferred to a solution of the primary antibody in TTBS and incubated for 1 hour. 1:1000 and 1:4000 dilutions of antibody were tested. The membrane was then washed in 3-4 changes of TTBS over 15 minutes with gentle agitation. The Vectastain ABC reagent was prepared by mixing reagent A (Avidin DH) with reagent B (Biotinylated Horseradish peroxidase H) as per the manufacturer's instructions and allowing 30 minutes for complex formation before use. The membrane was then transferred to a dilute solution of biotynylated secondary antibody (1:1000) in TTBS, and incubated for 30 minutes with gentle agitation. After another set of washes, the membrane was transferred to the ABC reagent for 30 minutes. The membrane was then washed thoroughly, transferred to a clean receptacle and developed using the substrate solution (diaminobenzidine (0.1% in 0.1M Tris buffer, pH 7.2) mixed with 0.02% hydrogen peroxide (in dH_20 from a 30% stock). After developing, the membrane was washed with 2 changes of distilled water over 10 minutes and allowed to dry. The nitrocellulose was then stored in the dark.

2.14 Aphid Infection Experiments

2.14.1 Rearing of *Myzus persicae*

Myzus persicae, the Peach Potato Aphid, was reared on Sweet Pepper under a strict light/dark regime of 16h light and 8h dark, temperature of 23°C. Stock cultures of

aphids were maintained in adapted Blackman boxes - a small box with a muslin 'window' housing a pepper leave moistened by a damp sponge, under the same conditions. Aphids from these stock cultures were used to inoculate fresh pepper plants in the cages (the infested leaf was laid gently onto a 'clean' plant). Fresh plants were added to the cultures weekly, and aphids from the older plants allowed to migrate onto them. Dying plants were routinely removed from the cultures. Periodically the cages were cleaned and a fresh colony cultured, to prevent overcrowding, and potential build up of pathogens. R1 clones of the aphid, which demonstrate partial resistance to pyrethroid and organophosphate insecticides (by means of esterase production) were used in all experiments to represent the natural population of peach potato aphid.

2.14.2 Inoculation of aphids

Chambers for incubation of insects were prepared as follows. The base of a 5cm Petri-dish was half filled with 1% distilled water agar. Sweet Pepper leaves were cut to fit the base of the dish and were placed, underside facing upwards, onto the water agar once it had cooled and was just about to set. This system maintained leaves in a hydrated and healthy condition over the course of the experiment.

Aphids were transferred to the leaf disks using a sparsely bristled paint brush. To avoid damage to mouth parts, aphids were lifted from the rear, and where possible, only moving aphids were taken. A thin layer of fluon® was painted around the top sides of each chamber to prevent escape of the aphids and the dish was then sealed

with perforated parafilm®. The parafilm®, in addition to sealing the dish, provided an almost 100% humid environment. collecting small droplets of moisture in the dish.

For each spore suspension of *V.lecanii* to be tested, estimated spore counts were made using a haemocytometer and the appropriate dilution in sterile dH_20 made. For *in vivo* detection of PR1-like activity 4 x 10^6 conidia per ml and for immunocytochemistry experiments 1 x 10^7 conidia per ml were used.

Replicate plates containing the aphids were then sprayed with 1500µl of each spore solution and control plates were sprayed with 1500µl sterile dH_20 using a Potter Spray Tower. The spray tower used has been calibrated so that for each spore concentration sprayed, the number of spores covering the surface as a whole, and on the individual aphid can be estimated (figure 2.2).

Surface	Estimation of spore number	Linear regression
Glass slide	log(y)=1.042log(x) - 4.896	$R^2 = 0.9079$
Aphid (dorsal)	log(y)=1.279log(x) - 6.783	$R^2 = 0.9441$
Aphid (ventral)	log(y)=0.882log(x) - 4.817	$R^2 = 0.7743$

Table 2.1. Calibration of Potter Spray Tower (Roditakis, University of Bath). Spore coverage could be estimated using the equations shown, whereby, (y) = conidia / mm^2 and (x) = conidia ml^{-1} sprayed. Linear regressions were fitted on the data from spore counts.

The parafilm® was replaced and the plates transferred to a growth cabinet with the same light dark cycle as the aphid culturing conditions, at 23°C. Plates were monitored daily for signs of mortality. An aphid that showed no movement after being inverted using the brush was deemed to be dead, and death was attributed to *V.lecanii* if these aphids were shortly seen to be sporulating.

2.14.3 Testing aphids for PR1-like activity

5 aphids from each replicate were transferred to individual wells of a micro-titre plate and homogenised using a multiple well homogeniser in 100µl 0.225M Tris-HCl pH8. 50µl of a 1mM solution of the fluorescent PR1 substrate, Suc-Ala-Ala-Pro-Phe-AMC (Calbiochem-Novabiochem AG) in dimethylsulphoxide was added to the homogenisation mix and the release of amino methyl-coumarin measured using a Fluoroskan Fluorometric plate reader over 1 hour. 5 replicates for each isolate and day of infection were used. Aphids sprayed with sterile dH_20 were also tested daily.

Homogenates for analysis by iso-electric focusing were supplemented with 5mM phenylthiol urea (PTU) to prevent clotting and melanisation of haemolymph. Samples were centrifuged at 11600g for 3 minutes to remove particulate matter and 75µg protein was loaded onto a broad range iso-electric focusing gel, 3cm from the cathode. Samples were focused as previously described and qualitatively assayed for protease activity using gelatin and for PR1-like activity by gel slice assays as described earlier.

2.15 Immunocytochemistry

2.15.1 Fixing and sectioning of aphids

Infected and control aphids to be fixed, were transferred into individual adapted 'Beem Capsules' (small containers with fine gauze at one end to minimise handling of the insect.) All stages were performed under vacuum. Each aphid was prefixed in 2.4ml glutaraldehyde, 0.2ml acrolein, 6.66ml 0.3M PIPES buffer (Piperazine-N,N'-bis [2-ethanesulphonic acid] and 0.1M Sodium Hydroxide) and 0.02g Calcium chloride for seven hours at room temperature. Pre-fixed aphids were then washed overnight in 0.2M PIPES buffer supplemented with 0.1% $CaCl_2$.

Aphids were postfixed in 0.15M PIPES buffer (pH 7.4) containing 1% Osmium tetraoxide and 0.1% $CaCl_2$ for 1 hour at room temperature. Dehydration of fixed aphids was performed in 3, 5 minutes washes of a series of increasing acetone concentrations (50, 70, 80, 95 and 100%). Fixed aphids were infiltrated with Spurr's resin by decreasing the acetone:resin ratio over 1 hour changes (3:1; 1:1; to 1:3) until aphids were left in 100% resin overnight. (Floating aphids indicated entrapment of air and thus poor penetration of the fixative). The next day, aphids were transferred to fresh resin for 6-7 h after which, they were again transferred to fresh resin, in a labelled rubber mould and the resin was allowed to polymerise at 70°C for 6 hours. Where possible, the aphid was orientated so that the anterior of the aphid was at the pointed end of the well.

Gelatinization of slides was performed prior to sectioning the aphids. Slides were washed repeatedly in detergent and several changes of dH_20 to ensure they were clean

before coating. 2.25g gelatin was dissolved in 800ml dH_20 at 70°C. Once cooled, 0.23g chromic potassium sulphate was added and the mixture stirred gently. A further 100ml dH_20 was added and the subbing solution was transferred to a glass trough. Slides to be coated were gently agitated in the solution and then left for 2 minutes. After allowing to dry overnight, slides were dipped into the gelatin solution once more for 1 minute then allowed to dry. Slides were stored at room temperature until required.

Thin sections for light microscopy were cut using a glass knife to 500nm thickness and ultra-thin sections for electron microscopy were cut using a diamond knife to 100nm. Sections were carefully transferred into a droplet of water on a treated slide and allowed to heat dry onto the slide.

2.15.2 Localisation of PR1-like enzymes within aphids using light microscopy

Immunostaining was performed using the Vectastain® ABC Kit as used for Western Blotting. Sections for immunohistochemical staining were transferred to 0.2M Phosphate Buffered Saline (PBS), pH8, the optimum for the PR1-like enzyme, for 20 minutes. All available sites were blocked with 15µl goat serum in 1ml PBS (enough for 10 slides), for 1 hour at room temperature. The excess was removed and primary antibody applied in a 1:2000 dilution in PBS supplemented with goat serum (15µl as previously). Slides were left overnight in primary antibody in a humid chamber to prevent drying.

Slides were then washed in 4 changes of PBS before transferring into a solution of biotinylated antibody (1:1000) in PBS containing 15µl goat serum for 1 hour. The ABC reagent was mixed 30 minutes before use. Slides were again washed in several changes of PBS before transfer into the ABC reagent for 1 hour. After the last set of rinses, the slides were developed in 2ml ImmunoPure® metal enhanced DAB substrate and 18ml hydrogen peroxide buffer (Pierce). Slides were monitored using a light microscope for between 2 and 10 minutes and the reaction was stopped by washing repeatedly in distilled water. Slides could also be counter stained in Epoxy tissue stain (Toluidine Blue and basic fuchin) for 30 seconds on a hot plate, followed by rinsing in distilled water. Slides were dried, mounted with Eukitt® and photographs were taken.

2.15.3 Localisation of PR1-like enzymes within aphids using electron microscopy

100nm sections for electron microscopy were mounted onto copper grids directly, without the use of plastic film, as this appeared to give a high background during immunogold labelling. The copper grids were carefully manipulated on a clean sheet of dental wax at all stages of immunostaining. Each grid was floated on a drop of TBST (as for light microscopy) placed on the dental wax for 15 minutes. Sections were blocked with 2% skimmed milk in TBST for 1 hour and then washed in three, 10 minutes, rinses of TBST. Sections were transferred to 1:100 dilution of primary antibody in TBS for up to 2 hours, and then were rinsed again in three changes of TBST before transferring to 1:100 dilution of secondary antibody-gold conjugate in TBS for 2 hours. Grids were washed in TBST followed by dH_20 before allowing to dry.

Prior to viewing under Electron Microscopy, sections were stained in uranyl acetate and lead citrate. Uranyl acetate stains nucleic acids (ribosomes, chromatin etc.) whilst lead citrate, a product of the reaction between lead nitrate and sodium citrate, indicates areas where the osmium tetraoxide has precipitated - unsaturated lipids, membranes etc. Both solutions were filtered prior to use. Copper grids were placed onto drops of fresh warm dH_20 for three minutes prior to staining. Grids were then removed from the water and excess moisture was removed using filter paper, each grid was then placed into a drop of uranyl acetate, covered and left for 10 minutes. Grids were then washed in repeated rinses of distilled water before transferring into lead citrate for 4 minutes. (Several crystals of sodium hydroxide surrounded to lead citrate to absorb environmental carbon dioxide). Grids were again washed thoroughly in dH_20 before viewing using the Electron Microscope.

3 Biochemical Studies On The Production And Mode Of Regulation of a PR1-like enzyme from five strains Of *Verticillium lecanii*

3.0 INTRODUCTION

The high level of production of proteinases in insect-pathogen interactions suggests an important function for these enzymes and is attracting much interest at present (St Leger et al. 1997 &1998). Investigating the mechanisms regulating the secretion of proteinases is important for the further understanding of pathogen growth and development *in vivo* (St Leger et al. 1998). By investigating the aspects that make an entomopathogenic fungus, different from a saprophyte or plant pathogenic fungus may further enhance our understanding (Gupta et al. 1994). These differences may operate by way of regulatory controls expressing genes under conditions in which similar genes in saprophytes are not expressed (St Leger et al. 1998).

Filamentous fungi are able to grow under a variety of environmental conditions and have evolved sophisticated regulatory mechanisms to allow them to detect and respond to changes in the environment. The efficient utilisation of nutrients is under the control of both wide-domain and pathway-specific regulatory genes (Hynes 1994). Work to date suggests that there is a strong selective advantage to express genes of a specific pathway only when a suitable substrate is available (Screen et al. 1997). Furthermore, studies of pathways mediating carbon catabolite and nitrogen metabolite repression to date indicate that such regulatory mechanisms may be widely conserved amongst fungi (Caddick et al 1994, Marzluf 1997; Arst 1995 and Ronne 1995).

A number of authors have suggested that patterns of enzyme production represent an adaptation to the polymers present in the integuments of their particular hosts (St Leger et al. 1997, Gupta et al. 1994). As a result specialised pathogens isolated from either plant or insect hosts may be less versatile than the opportunists (Gupta et al. 1994). The mode of induction of PR1 from *M.anisopliae*, for example, has been studied in detail and suggests adaptations to insect parasitism (Clarkson and Charnley 1996).

PR1 and PR2 from *M.anisopliae* appear to demonstrate different levels of induction. PR2 is induced by a range of proteins from insoluble cuticle, elastin and collagen as well as gelatin and bovine serum albumin, BSA (Paterson et al. 1993). Production of PR1 was found to be repressed in BSA containing cultures but considerably enhanced on insect cuticle, leading the authors to conclude that this enzyme is specifically induced by some component(s) of the insect cuticle (Paterson et al. 1994a). Other proteases such as aminopeptidase and propyl-dipeptidylpeptidase also appear to be induced by insect cuticle (St Leger et al. 1993a), again apparently reflecting the adaptation of *M.anisopliae* to insect parasitism. Further work has indicated that the inducing components of the cuticle are proteinaceous and that PR1 production also appears to show a level of induction by potential breakdown products of the host cuticle, such as amino acids (Paterson et al. 1994b).

Protease regulation in fungi can be controlled by both induction and derepression mechanisms (reviewed by Cohen 1980). In fact, many extracellular fungal enzymes,

particularly protease, chitinase and GlcNAg-ase are subject to catabolite repression (Bidochka and Khachatourians 1988a,b; St Leger et al. 1986c; 1988c). Studies in *M.anisopliae* indicate that these C and N de-repression mechanisms operate at the level of transcription (St Leger et al. 1991c, 1992c).

A number of lines of evidence suggest that PR1 is produced under conditions of carbon and nitrogen starvation (St Leger et al. 1988c, 1991c and 1995b). Recently, the genomic *pr1* promoter region of *M.anisopliae* was sequenced and found to contain several putative binding sites for regulatory proteins similar to the carbon-catabolite repressor (CREA) and nitrogen-metabolite regulator (AREA) of *Aspergillus nidulans* (Screen et al. 1997). Paterson et al. (1994a) found that maximum levels of PR1 were produced only when the fungus was grown on insect cuticle as the sole source of carbon and nitrogen, but although a level of repression was observed by carbon, PR1 was predominantly repressed by nitrogen. A basal level of PR1 synthesis (that is, in the absence of cuticle) was also observed (St Leger et al. 1995b).

There appears to be co-ordinate regulation of appressorium formation and PR1 production. The addition of readily utilisable nutrients to host cuticle not only reduced enzyme production but also penetration of the cuticle (St Leger et al. 1989b). Thus nutrient starvation is likely to be a key environmental signal for the switch from a saprophyte to a pathogenic mode of growth (Clarkson and Charnley 1996). Catabolite repression could also operate if the release of degradation products from the cuticle exceeded fungal requirements presumably conserving production of the enzyme in environments where it is not needed. Given this dependence on the environment, other

factors ensuring the productive action of extracellular enzymes, such as pH are also likely to be important (St Leger et al. 1998).

Enzyme production, as a part of the pathogenic process of this fungus. appears to only occur when a lack of extracellular nutrients and depletion of endogenous reserves make it necessary for the pathogen to establish a nutritional relationship with the host (St Leger 1995).

PR1-like enzymes may differ between isolates. Leal et al. (1997) actually used differences between *pr1* genes to identify and characterise different strains of *M.anisopliae*. In this study, protease production by 5 strains of *V.lecanii*, has been investigated on insect cuticle. Extensive studies have been conducted in order to identify differences in regulation between PR1-like enzymes produced by these strains. Iso-electric focusing studies have been conducted on those isolates displaying interesting differences of subtilisin production with respect to induction and catabolite repression.

3.1 RESULTS

3.1.1 Nomenclature and Morphology of Isolates of *Verticillium Lecanii* Investigated

5 isolates of *Verticillium lecanii*, provided by Koppert B.V., the Netherlands, were used in this study. KV01 and KV71 are the isolates used in Koppert's commercial formulations, Mycotal™ (for whitefly) and Vertalec™ (for aphids) respectively. For ease of reference, their commercial names are used in this thesis.

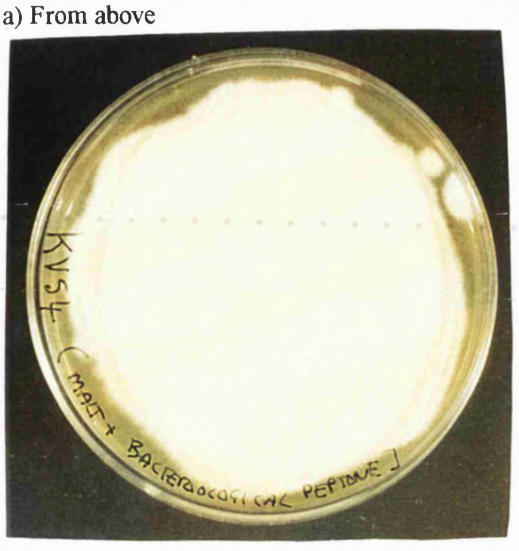
a) Growth on Solid Media

Figure 3.1. shows the growth after 10 days of all 5 isolates on the two types of agar routinely used throughout the study after 10 days. On the rich medium, malt plus bacteriological peptone, all strains grow as a thick mat of white mycelium upon which the conidia are born. The plate, when viewed from below, appears to be a dark golden yellow. Isolates display a very similar rate of growth to each other on this medium. When cultured on just malt agar, growth relative to that on the rich medium is sparse for all strains. KV54 and Vertalec appear to grow the most quickly on this medium producing a more feathery appearance over the plate. For KV42, KV22 and Mycotal, mycelial growth is minimal although more evenly distributed across the plate than KV54 and Vertalec. The base of all malt plates did not appear to show any darkening relative to that of the original colour of the medium.

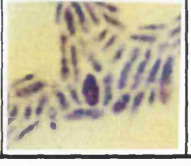
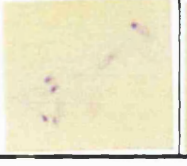
Figure 3.1. also shows the conidia produced on these two types of solid media as stained with Toluidine blue. For all isolates of *V.lecanii* examined, the conidia formed on solid media were long and thin in shape and approximately 2.5μm in length. Differences between conidia of different isolates were observed in the actual staining of the conidia, for KV22, in particular, incomplete staining resulted in only the tips of each spore being visible. There did not appear to be any size or shape differences on the two different media.

Figure 3.1. Strains of *Verticillium lecanii* used throughout studies and their growth on solid media.

a) Isolate KV54 (as example of solid growth, morphology similar between strains)

Malt + bacteriological peptone	Malt agar
a) From above	a) From above
	
b) From below	b) From below
	

b) Conidia isolated from two different media, for five isolates of *V.lecanii*

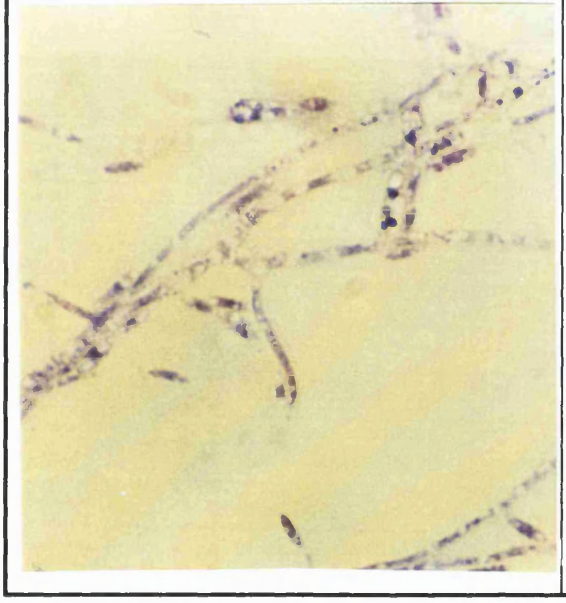
Conidia	Mycotal (KV01)	Vertalec (KV71)	KV42	KV22	KV54
From Malt					
From Malt + bacteriological peptone					

_____ (Bar represents 10μm)

b) Growth in Liquid Culture

When grown for four days at 23°C, shaking at 120rpm in a liquid complete medium, cultures of *Verticillium lecanii* usually have a very thick creamy consistency. On closer inspection, growth appeared to be as a mixture of mycelia, hyphal fragments and spores. Vertalec and KV22, however, appeared to display an occasional switching of morphology between two extremes. Without any apparent change to the culture conditions, they have been observed to grow as discreet pellets, principally as mycelia and as a very thin consistency, principally as spores. Figure 3.2. shows these two forms. For all regulation experiments, strains were used when growing under the more common form, of a thick creamy texture.

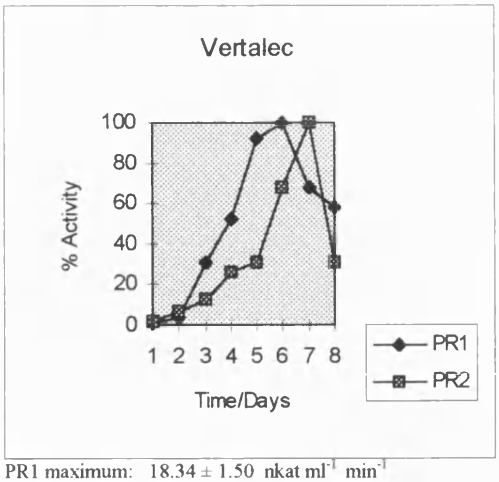
Figure 3.2. Morphology of Vertalec when grown in complete liquid media

a) Mycelia and hyphal fragments - as retained on the muslin filter	b) Spores - as allowed to pass through muslin filter, larger in width than conidia found in solid media but not spherical in shape as blastospores
	

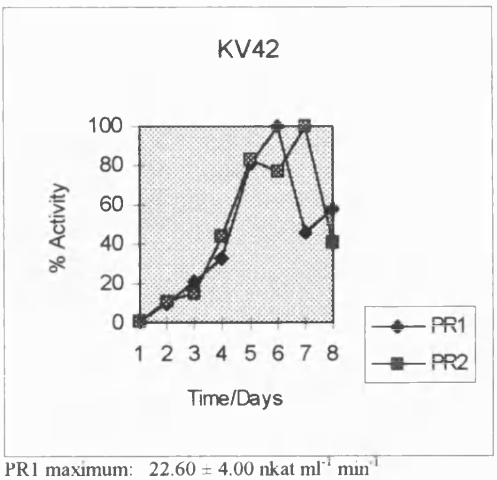
_____ (bar represents 10μm)

3.1.2 Production of PR1-like and PR2-like enzymes by 5 strains of *V.lecanii* on locust cuticle over time

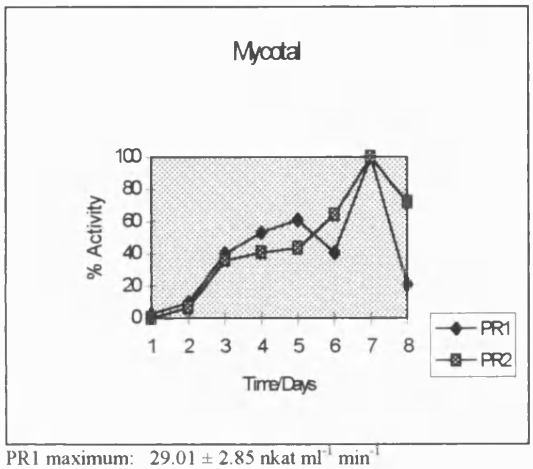
Production of PR1 and PR2-like enzymes on locust cuticle, as the sole source of carbon and nitrogen, was monitored over time for 5 isolates of *V.lecanii* (figure 3.3). Proteinase activity (both PR1 and PR2) appeared in cuticle cultures 2d after inoculation. The production of PR1-like enzymes peaked at day 6 in Vertalec, KV42 and KV22 cultures, Mycotal later at day 7. KV54 displayed two peaks at 4 and 7 days. PR2-like activity peaked one day later than the chymoelastase enzyme in Vertalec, KV42 and KV54 cultures and simultaneously in KV22 and Mycotal cultures. In this experiment highest PR1 and PR2-like activities were seen in Mycotal and KV42 cultures respectively. For all isolates, however, peak PR2-like activity was lower than that of peak PR1-like activity over the time course.



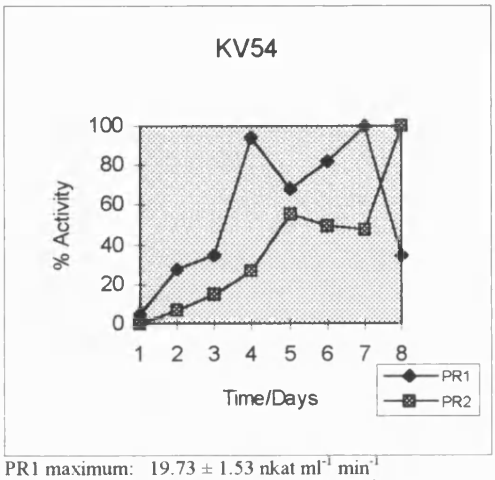
PR1 maximum: 18.34 ± 1.50 nkat ml^{-1} min^{-1}
PR2 maximum: 15.14 ± 1.53 nkat ml^{-1} min^{-1}



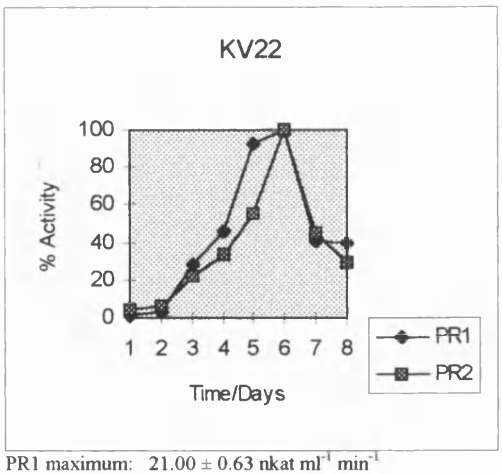
PR1 maximum: 22.60 ± 4.00 nkat ml^{-1} min^{-1}
PR2 maximum: 18.34 ± 2.68 nkat ml^{-1} min^{-1}



PR1 maximum: 29.01 ± 2.85 nkat ml^{-1} min^{-1}
PR2 maximum: 9.87 ± 1.25 nkat ml^{-1} min^{-1}



PR1 maximum: 19.73 ± 1.53 nkat ml^{-1} min^{-1}
PR2 maximum: 8.74 ± 0.92 nkat ml^{-1} min^{-1}



PR1 maximum: 21.00 ± 0.63 nkat ml^{-1} min^{-1}
PR2 maximum: 12.34 ± 1.45 nkat ml^{-1} min^{-1}

Figure 3.3: PR1-like and PR2-like production is shown over a time course of 8 days for five strains of *Verticillium lecanii* grown on locust cuticle. Enzymic activity is expressed as a percentage of the maximum activity. Actual maximum activity, shown in nkat NA released ml^{-1} min^{-1}, is shown beneath each graph. Mean values were calculated from 3 replicate flasks, standard deviations are also shown.

3.1.3 Studies on the mode of regulation of PR1 and PR2-like enzymes by isolates of *Verticillium lecanii.*

3.1.3.1 The effect of different sources of carbon and nitrogen on the production of PR1-like and PR2-like enzymes by Vertalec.

A four day old culture of Vertalec, grown in complete media, was starved for 24 hours in a basal salts medium lacking in both carbon and nitrogen. After this period of starvation, the biomass was transferred to media containing different carbon and nitrogen sources (supplied at 1% w/v) and PR1-like activity monitored over time (Figure 3.4.)

Cellulose, gelatin, urea and xylan did not support PR1-like enzyme production by Vertalec over 24 hours. At 8 hours, subtilisin production was hardly detectable in practical grade chitin containing cultures and significant in cuticle containing cultures. Significant levels of PR1-like activity were also observed on Bovine Serum Albumin (BSA). By 16 hours, levels of PR1-like activity were high in cuticle containing cultures but also substantial in chitin, BSA and KOH treated chitin (i.e. deproteinised-chitin) containing cultures. Low levels of activity were observed on elastin. At 24 hours maximal levels of PR1-like activity were found in deproteinised chitin cultures, comparable with those on cuticle. Activity levels in BSA cultures were approximately 40% of that of cuticle cultures. Enzyme activity was also observed on chitin, with low levels in elastin and control cultures (basal salts alone).

Filtrates from these cultures were also tested against the PR2-like substrate, N-Bz-Phe-Val-Arg-pNA, however only very low levels of activity were found for the 24 hour duration of the experiment (< 0.8 nkat $ml^{-1}min^{-1}$) in all cases. The experiment was thus extended to 32 hours (Figure 3.5.) These results indicate that PR2-like enzymes of this strain of *V.lecanii* are produced later than the PR1-like enzymes. Low levels of PR2 were observed in all culture conditions at 24 hours with the exception of the control and urea. Cuticle, Bovine Serum Albumin and gelatin showed substantial PR2-like activity at 24 hours. At 32 hours, insect cuticle, elastin and BSA containing cultures contained comparatively high levels of PR2-like activity, although still at least four fold lower than peak PR1-like activities.

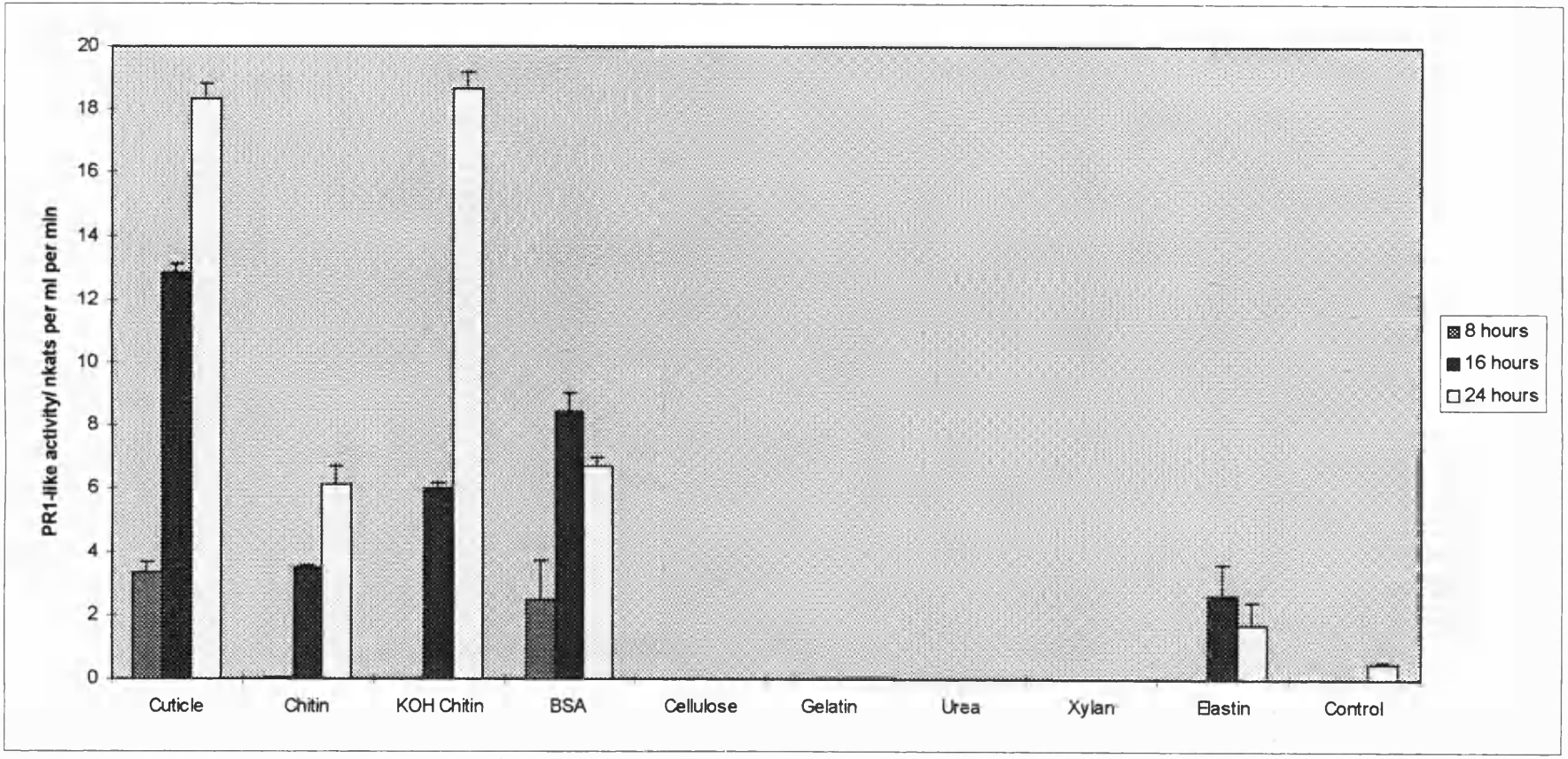


Figure 3.4. PR1-like activity produced by Vertalec on different sources of carbon and nitrogen 8, 16 and 24 hours after transfer. Enzyme activities are expressed as nkat ml^{-1} min^{-1} from N-Suc-Ala-Ala-Pro-Phe-pNA from 3 replicate flasks, standard deviations are shown.

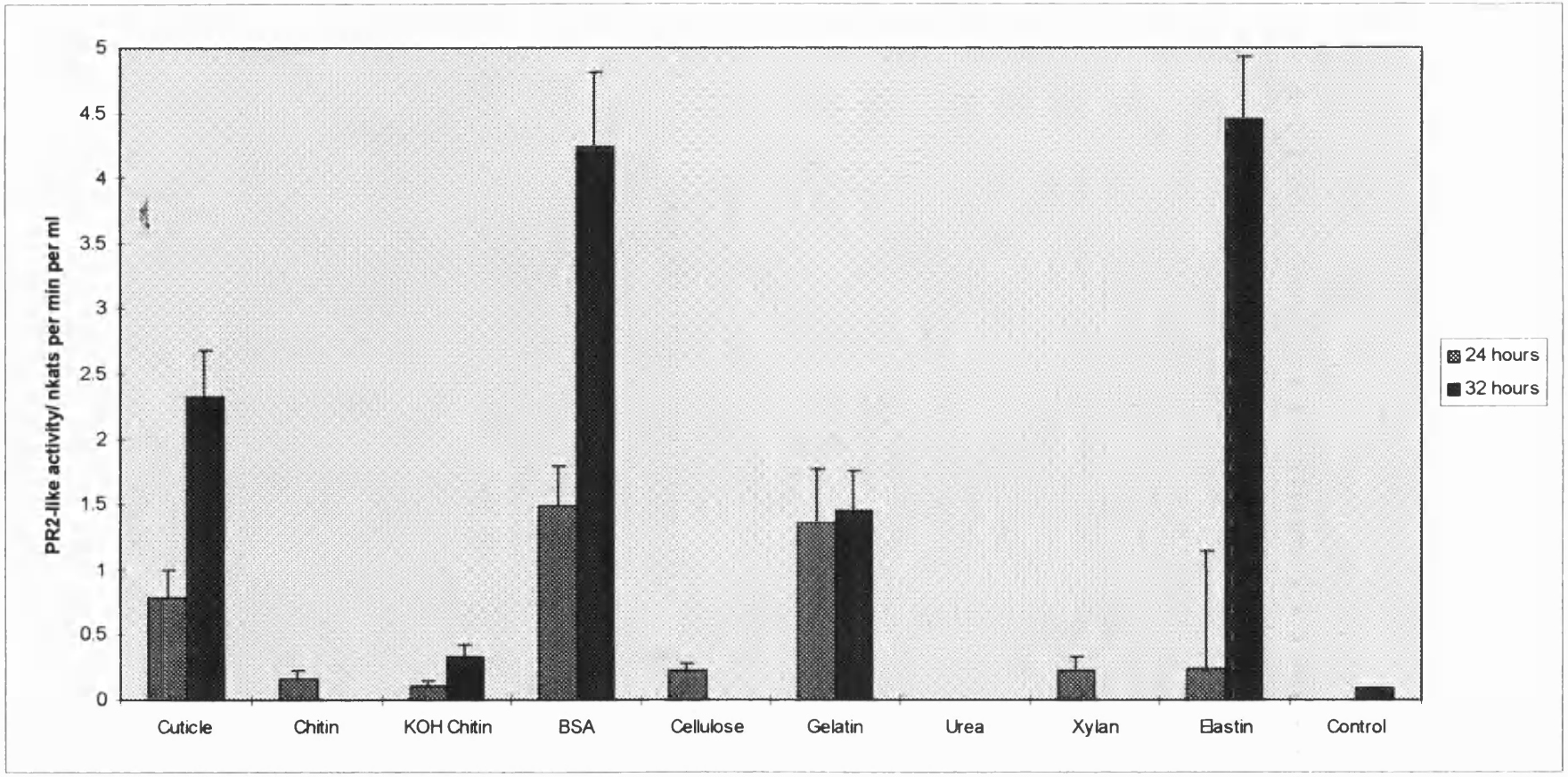


Figure 3.5. PR2-like activity produced by Vertalec on different sources of carbon and nitrogen 8, 16 and 24 hours after transfer. Enzyme activities are expressed as nkat ml^{-1} min^{-1} from N-Bz-Phe-Val-Arg-pNA from 3 replicate flasks, standard deviations are shown.

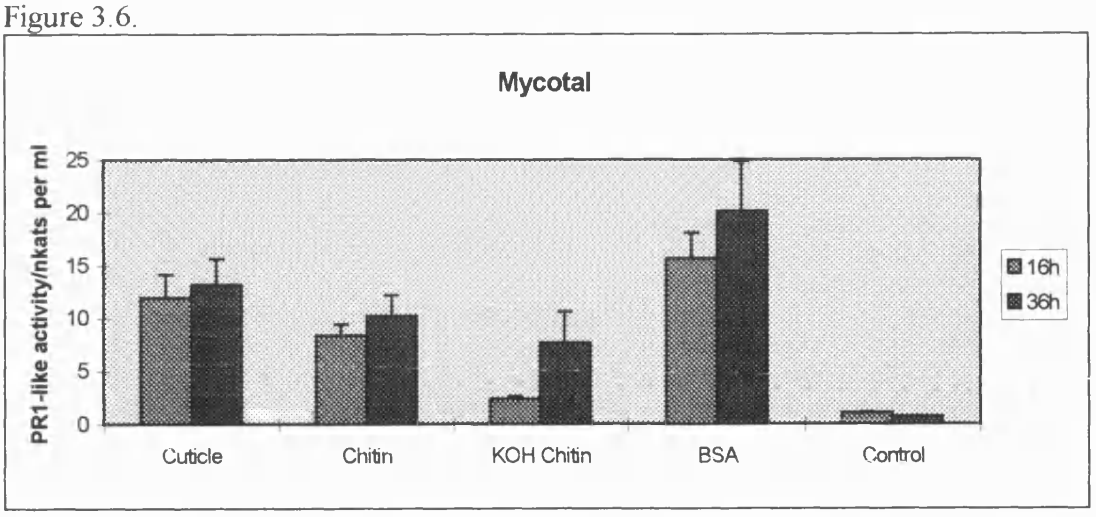
3.1.3.2 The effect of different sources of carbon and nitrogen on the production of PR1 and PR2-like enzymes by 5 isolates of *V.lecanii*

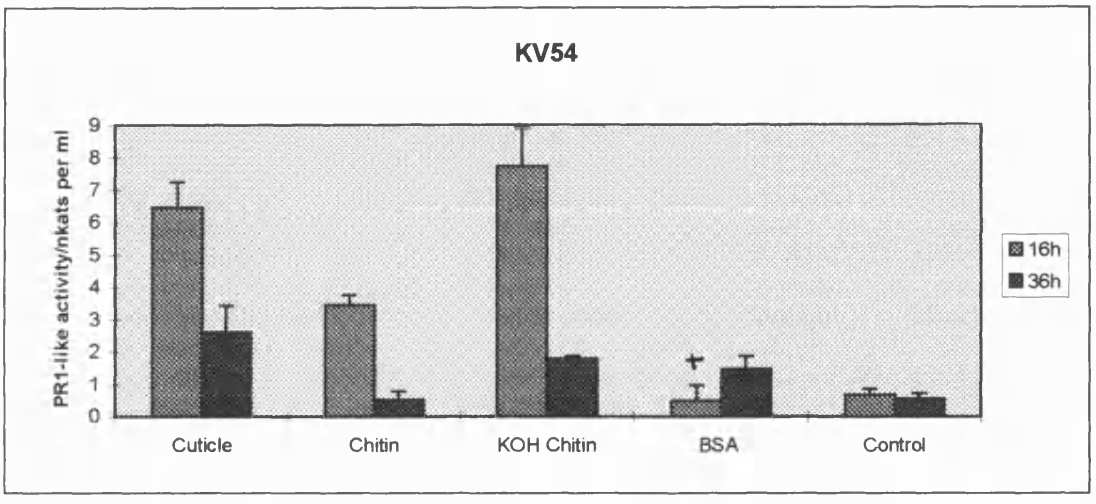
PR1-like activity of Vertalec was produced optimally on insect cuticle and deproteinised chitin. The study was extended to a further 4 isolates of *V.lecanii* to determine whether the situation with Vertalec was typical of the species. Figure 3.6. shows PR1-like activity 16 and 36 hours after transfer into five substrates for each strain of *Verticillium lecanii*. The substrates tested (supplied at 1% w/v) were locust cuticle, practical grade chitin (from crab shells), deproteinised chitin (KOH treated chitin), Bovine Serum Albumin (BSA) and basal salts alone (control).

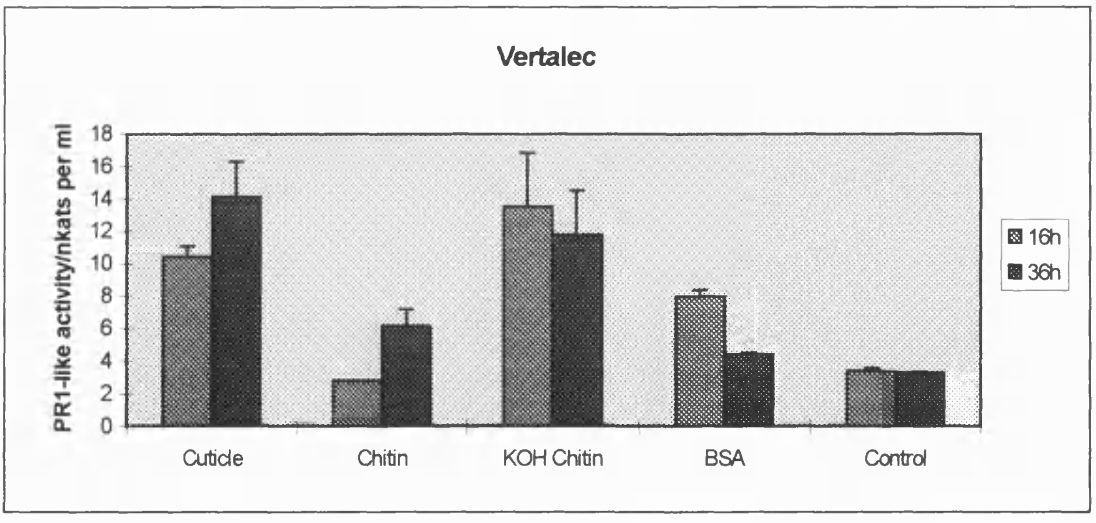
Statistical comparisons of data were performed using a Oneway Analysis of Variance, testing the null hypothesis that the enzyme activities were from the same source. At 4,75df, the probability of the treatments being the same at 16 or 36 h was found to be less than 0.000 for each isolate.. Fisher's pairwise comparisons highlighted where treatments were significantly different from each other and the actual results of these statistical analyses are shown in the Appendices. (NB: '+' marks any treatments not significantly different from the control at that time point).

All isolates of the fungus tested produced significantly higher levels of chymoelastase activity in cultures containing cuticle, than in basal salts alone at 16 and 36 h. However, interesting differences in enzyme production on the other substrates were noted between isolates.

Figure 3.6.


Mycotal
PR1-like activity/nkats per ml
25
20
15
10
5
0
Cuticle
Chitin
KOH Chitin
BSA
Control
16h
36h


KV54
PR1-like activity/nkats per ml
9
8
7
6
5
4
3
2
1
0
Cuticle
Chitin
KOH Chitin
BSA
Control
16h
36h


Vertalec
PR1-like activity/nkats per ml
18
16
14
12
10
8
6
4
2
0
Cuticle
Chitin
KOH Chitin
BSA
Control
16h
36h

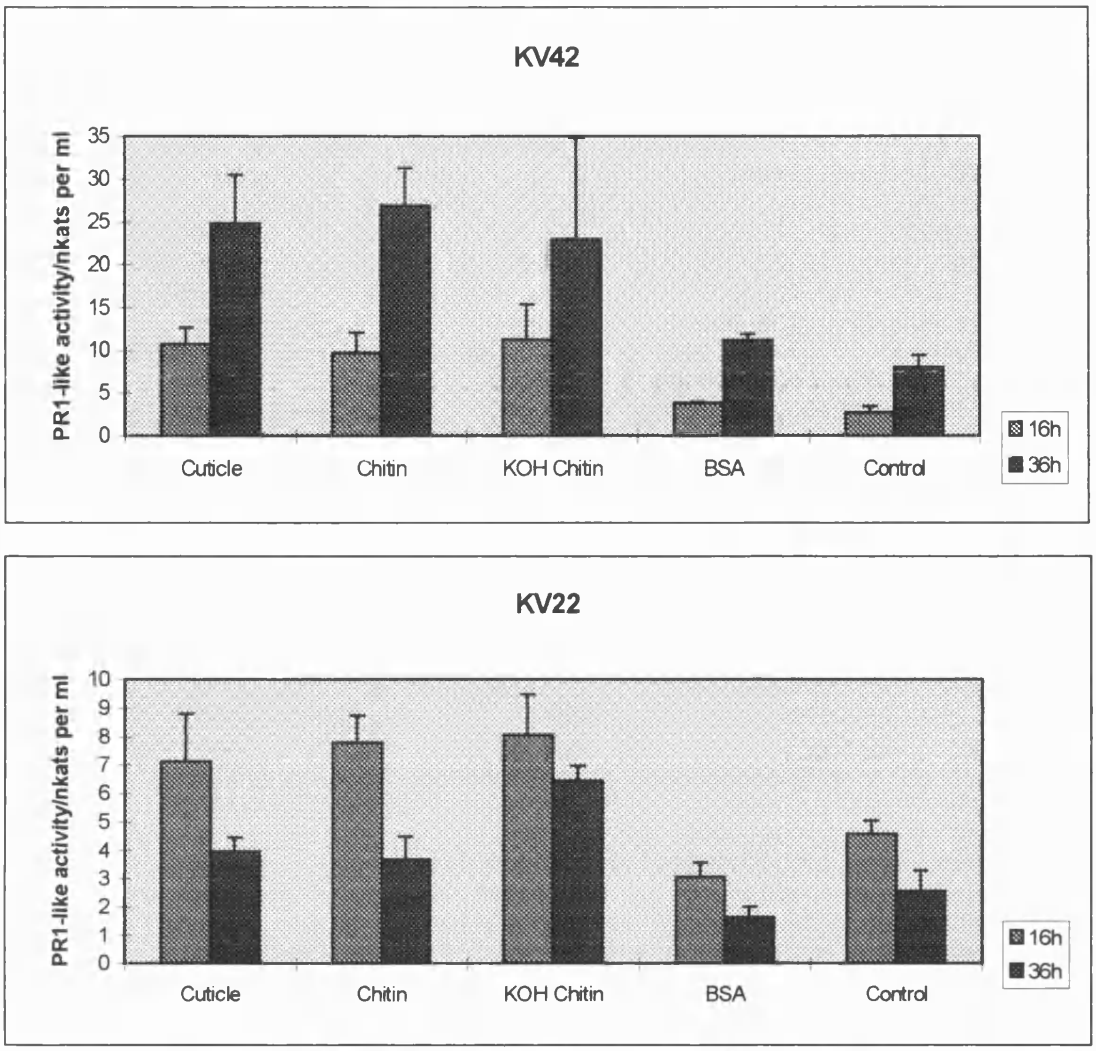


Figure 3.6. 4 day old fungus, grown in complete medium was starved for 24 hours before transfer to cultures containing either 1% locust cuticle, chitin, KOH treated chitin (deproteinised chitin), Bovine Serum Albumin (BSA) or just basal salts (control). Enzyme activity against N-Suc-$(Ala)_2$-Pro-Phe-pNA was recorded for each of the five strains shown on these substrates at 16 and 36 h post transfer. Results in nkat NA released min^{-1} ml^{-1} are shown as the mean of 4 replicates. Standard deviations are also shown. [+ = not significantly different from corresponding control].

At 16h, consistent with the previous experiment, Vertalec produced maximal levels of the protease in cultures containing crab shell chitin, treated with potassium hydroxide,

to remove any protein present. The order of enzyme production was KOH chitin > cuticle > BSA > chitin > control. The situation at 36 h was essentially the same.

At 16 hours, isolate KV54 displayed a similar pattern to Vertalec. Maximal levels of subtilisin activity were produced on KOH treated chitin, closely followed by cuticle. Practical grade chitin also supported substantial enzyme activity while BSA did not. Control levels were very low. In this instance chitin, unmasked by protein produced maximum enzyme activity.

KV42 and KV22 also produced PR1-like enzymes in cuticle and chitin containing cultures, however at 16 h activity was in the order cuticle = chitin = KOH treated chitin. In parallel with Vertalec at 36 h, KV22 exhibited maximal production on deproteinised chitin. Again. the protein BSA did not support PR1-like activity. Notably, isolate KV22 produced significantly more proteases in control cultures than on BSA.

The chymoelastases of KV42 and KV54, although under the same apparent control, exhibited a very clear difference in production over time. For KV42 there was a marked increase in enzyme activity from 16 to 36 hours in all cultures, however in KV54 cultures of cuticle, chitin and KOH chitin, production showed a very sharp decrease in activity from 16 to 36 hours.

In common with the other four isolates, Mycotal produced significant levels of PR1-like activity on cuticle, however its response to other substrates suggested a very

different regulatory control. Maximal activity at 16 and 36 hours was obtained in cultures containing the protein Bovine Serum Albumin. Cultures containing practical grade chitin did support protease production to a greater extent than the controls, however activity was significantly lower than in cuticle or BSA containing cultures. Activity in KOH treated chitin containing cultures appeared to be repressed until 36 hours. Thus Mycotal appears to produce serine proteases in response to protein whereas the other four isolates appear to be more sensitive to deproteinised chitin.

The peak levels of PR1-like activity in cultures of these five isolates were also markedly different. Mycotal and KV42 produced the most chymoelastase (~25nkat ml^{-1} min^{-1}), Vertalec produced medium levels of the enzyme (~14nkat $ml^{-1}min^{-1}$), and KV54 and KV22 were found to be quite low producers of the enzyme (~8nkat ml^{-1} min^{-1}). It should also be noted that similar levels of enzyme activity were observed before and after dialysis against water, showing that differences between isolates and between the same isolate on different media were not due to low molecular weight inhibitors.

3.1.3.3 The use of the fungal sterol 'Ergosterol' as a measure of fungal biomass in regulation experiments

Enhanced production of protease on certain substrates noted in the previous sections could denote regulation by induction or growth related enzyme synthesis. Since test conditions included insoluble substrates, direct measurement of fungal dry weight was not possible. Thus the quantification of the fungal sterol, ergosterol, was used as an

indirect measure of fungal biomass. Graystone (unpubl.) showed a linear relationship between ergosterol content and fungal dry weight in cultures of *V.lecanii*.

Vertalec was grown for 4d in complete medium then starved for 24 h in basal salts. Equal amounts of biomass (by weight) were transferred into each of the test cultures described previously. Ergosterol was extracted from the mycelium and analysed by HPLC (as described in Chapter 2). The chromatograms showed peaks with the same retention time as the ergosterol standard, and there were no interfering peaks. A calibration graph of ergosterol vs dry weight for 4 day old Vertalec (appendix) was used to estimate actual mass of the fungus in each culture.

By using ergosterol as a measure of biomass, it would appear that on transfer of equal quantities of Vertalec to each induction flask, there were no significant differences between different flasks in the amount of biomass present at 24 hours (Figure 3.7). This indicates that differences in protease production in these flasks could not be accounted for by differences in growth rate.

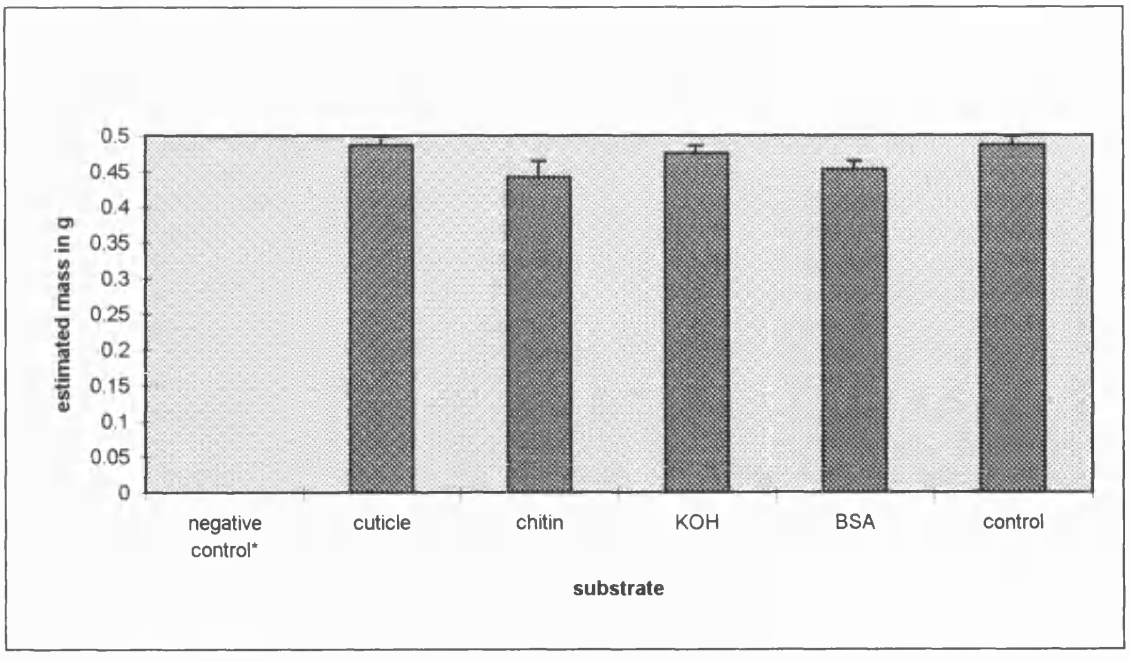
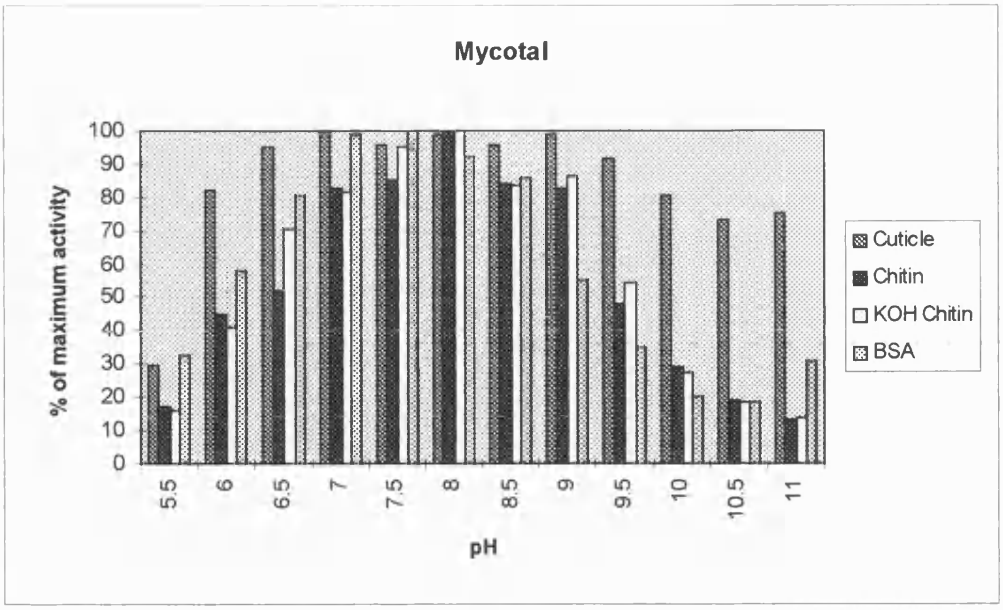


Figure 3.7.. Mean estimated biomasses of two replicate extractions of Vertalec from each induction flask, 24 hours post transfer. (Bars represent the maximal estimated biomass of the two extractions).

[* negative control: no fungal biomass was transferred into substrate flask. Extraction performed on basal salts to ensure no residual sterols were present]

3.1.3.4 pH optima of proteases from culture filtrates from regulation experiments

Culture filtrates of Mycotal and Vertalec were assayed for PR1-like activity at a range of pH conditions (figure 3.8) to determine the pH optima of protease produced by these isolates on different nutrient sources.



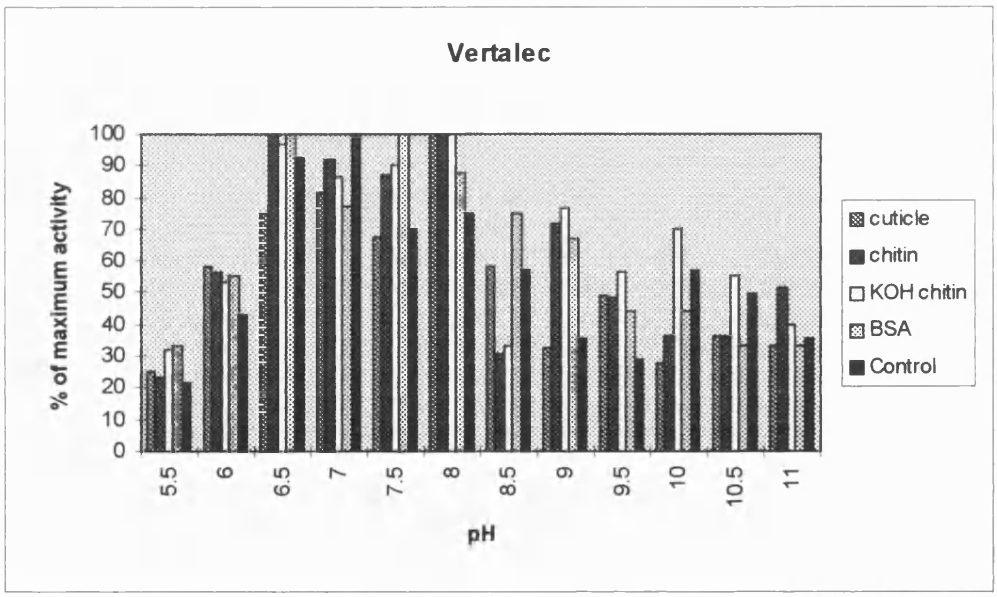


Figure 3.8. Culture filtrates from each of the test conditions for Mycotal and Vertalec were assayed for PR1-like activity at pH values between pH 5.5 and 11.0 under normal test conditions. Activity is expressed as a percentage of the maximum mean activity from 4 replicate flasks.
NB No values are expressed for control cultures of Mycotal since enzyme activity was negligible at all pH values.

Cuticle cultures of Mycotal were active against the chymoelastase substrate N-Suc-Ala-Ala-Pro-Phe-pNA over a broad pH range, highest activity was found between pH 7 and pH 9. Low pH appeared to be more inhibitory than high pH for these PR1-like enzymes. Filtrate from chitin and KOH treated chitin cultures was most active at

pH8.0 and did not hydrolyse the subtilisin substrate well at either extreme of pH. Protease activity from BSA cultures of Mycotal was still high at pH8.0 but appeared to be maximum at pH7.5 and again it was inhibited at extremes of pH.

Cultures of Vertalec on cuticle, chitin and deproteinised chitin, again, hydrolysed the substrate best at pH8.0, PR1-like activity being lower at very high or low pH. Filtrate from BSA and control cultures of this strain functioned well at pH8.0 against the PR1 substrate, however peak activity was found at slightly lower pH - pH7.5 and 7.0 for BSA and control cultures respectively.

3.1.3.5. The effect of N-acetyl glucosamine, when supplied in a restricted manner, on production of PR1-like enzymes by 2 lead strains of *Verticillium lecanii*

Maximum PR1-like protease activity in cultures of Vertalec occurred on deproteinised chitin. This may reflect specific induction by chitin or, more likely, a breakdown product of this polysaccharide. To investigate this, a comparison was made with Mycotal, where enzyme activity is not promoted by chitin, of the effect of N-acetyl glucosamine (the monomer of chitin). To prevent catabolite repression and to simulate *in vivo* conditions where N-acetyl glucosamine is present in restricted quantities, it was important to regulate substrate flow into the surrounding medium by means of diffusion capsules. The number of membranes covering the aperture of the capsule and the concentration of the GlcNAg were manipulated to adjust the rate of flow (figures 3.9 and 3.10).

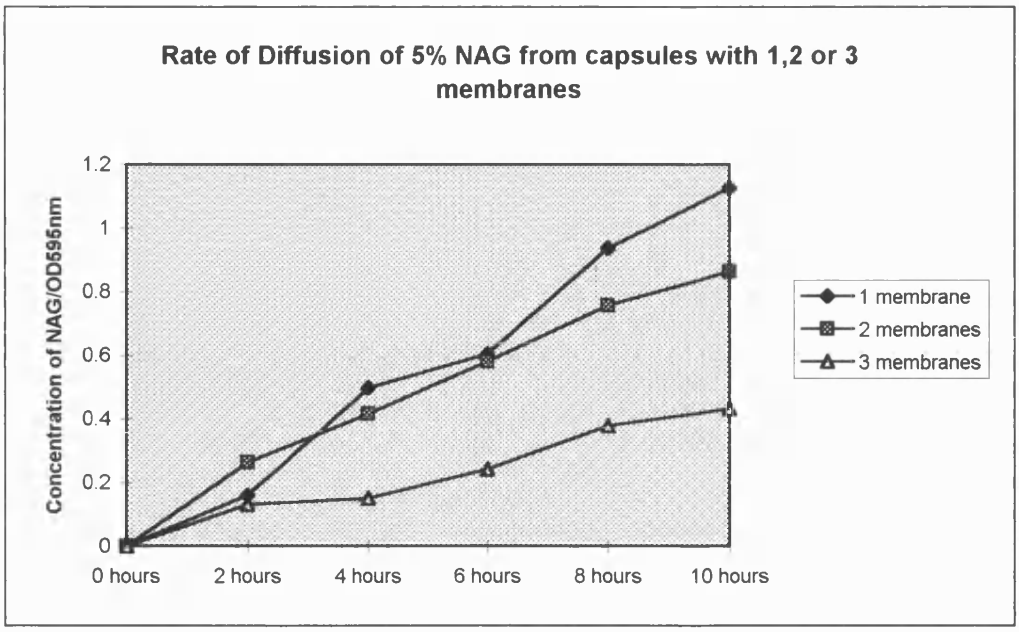


Figure 3.9. Diffusion capsules containing 5% GlcNAg were supplied to 100ml basal salt cultures which were transferred to a rotary incubator, set rotating at 120rpm. The rate of release of GlcNAg was controlled by either 1, 2 or 3 dialysis membranes covering the aperture of the capsule. The concentration of GlcNAg is represented by the optical density at 595nm. Rates of increase in optical density were calculated and a standard curve of GlcNAg used to calculate actual rate of diffusion of GlcNAg.

Estimated rates of diffusion from capsules, as calculated from the mean gradient, and N-acetyl glucosamine standards were as follows:

5% GlcNAg / 1 membrane: 24µg GlcNAg released ml^{-1} h^{-1}

5% GlcNAg / 2 membranes: 18µg GlcNAg released ml^{-1} h^{-1}

5% GlcNAg / 3 membranes: 13µg GlcNAg released ml^{-1} h^{-1}

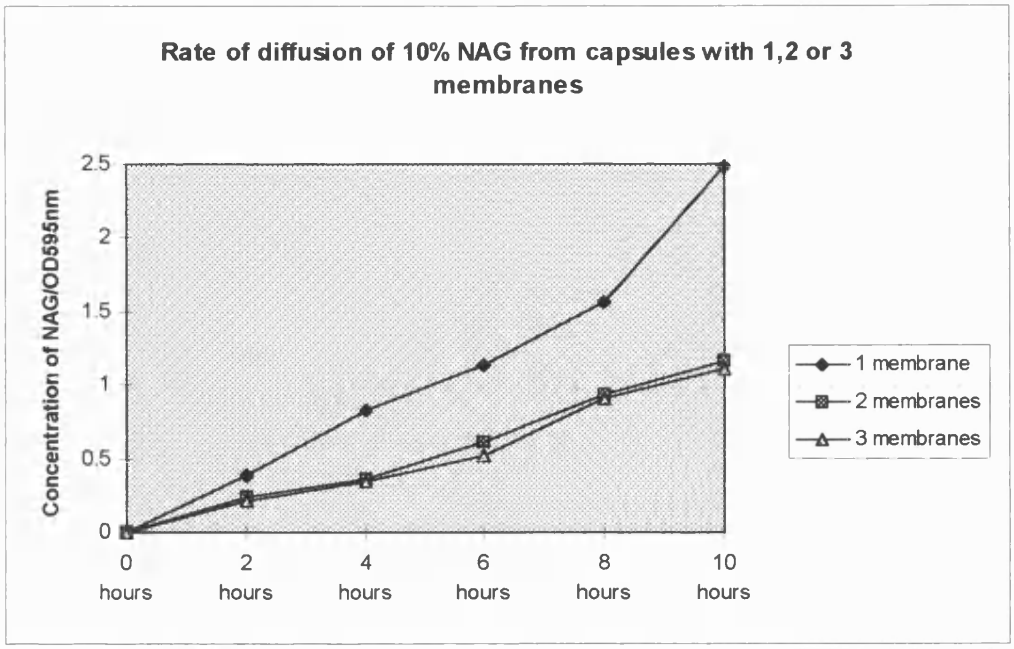


Figure 3.10. Diffusion capsules containing 10% GlcNAg were supplied to 100ml basal salt cultures which were transferred to a rotary incubator, set rotating at 120rpm. The rate of release of GlcNAg was controlled by either 1, 2 or 3 dialysis membranes covering the aperture of the capsule. The concentration of GlcNAg is represented by the optical density at 595nm. Rates of increase in optical density were calculated and a standard curve of GlcNAg used to calculate actual rate of diffusion of GlcNAg.

Estimated rate of release of N-acetyl glucosamine from capsules as calculated using the mean gradient and GlcNAg standards:

10% GlcNAg / 1 membrane: 36µg GlcNAg released ml^{-1} h^{-1}

10% GlcNAg / 2 membranes: 23µg GlcNAg released ml^{-1} h^{-1}

10% GlcNAg / 3 membranes: 21µg GlcNAg released ml^{-1} h^{-1}

Near linear rates of flow were obtained with both concentrations of N-acetylglucosamine. Cultures of Vertalec were assayed for free sugars under each of the flow rate conditions described over time relative to that of the sugar content of control flasks. With a flow rate of approximately 20-25µg GlcNAg ml^{-1} h^{-1}, (i.e. 5% GlcNAg and 1 membrane or 10% GlcNAg and 3 membranes) build up of sugar within

the medium was minimal (a mean increase of 1.5 ± 1.1 μg GlcNAg ml^{-1} h^{-1} in the culture as monitored over 16 hours). At a higher flow rate of 36μg GlcNAg released ml^{-1} h^{-1} (10%, 1 membrane), a mean increase of 4.02 ± 1.9 μg GlcNAg released ml^{-1} h^{-1} was observed, indicating that the rate of release of the monomer was greater than the rate of utilisation by the fungus. At a lower flow rate, observed with 5% GlcNAg and 3 membranes, there was an overall decrease of 6.3 ± 2.1 μg GlcNAg released ml^{-1} h^{-1}, suggesting that the rate of supply of the monomer was not as fast as that of its utilisation. As a result of these findings, for this study, **5% GlcNAg and 1 membrane** were used (the highest flow rate that could be utilised by this strain of *V.lecanii*).

To assess the effect of N-acetyl glucosamine (GlcNAg) on production of PR1-like activity by Vertalec and Mycotal isolates of *Verticillium lecanii*, it was necessary to include a number of positive and negative controls (figure 3.11). This was important to ensure that the isolates had retained the properties observed in earlier regulation experiments and to establish that elevated levels of protease activity were not due to the capsule itself. Firstly, it is important to note that for both Vertalec and Mycotal, peak PR1-like activity, as observed on insect cuticle, was considerably higher than in previous experiments. For Vertalec, control activity (on basal salts alone) was also high, although still approximately 2.5 fold lower than that found on insect cuticle (as similarly observed in previous experiments). Assay conditions and the source of the strains had not been altered from previous experiments, although glycerol stocks of the fungus were older.

The production of PR1-like enzymes by Mycotal was not altered by the presence of N-acetyl glucosamine supplied in a restricted or a non restricted manner. For Vertalec, PR1-like activity 16h after presenting established biomass with a capsule containing 5% N-acetyl glucosamine and 1 dialysis membrane was comparable to that found on insect cuticle and was significantly greater than that on controls. The capsule itself appeared to have a stimulatory effect, possibly by improving aeration, though the dialysis membrane did not. Direct supply of N-acetyl glucosamine did not promote PR1 production at either of the concentrations used.

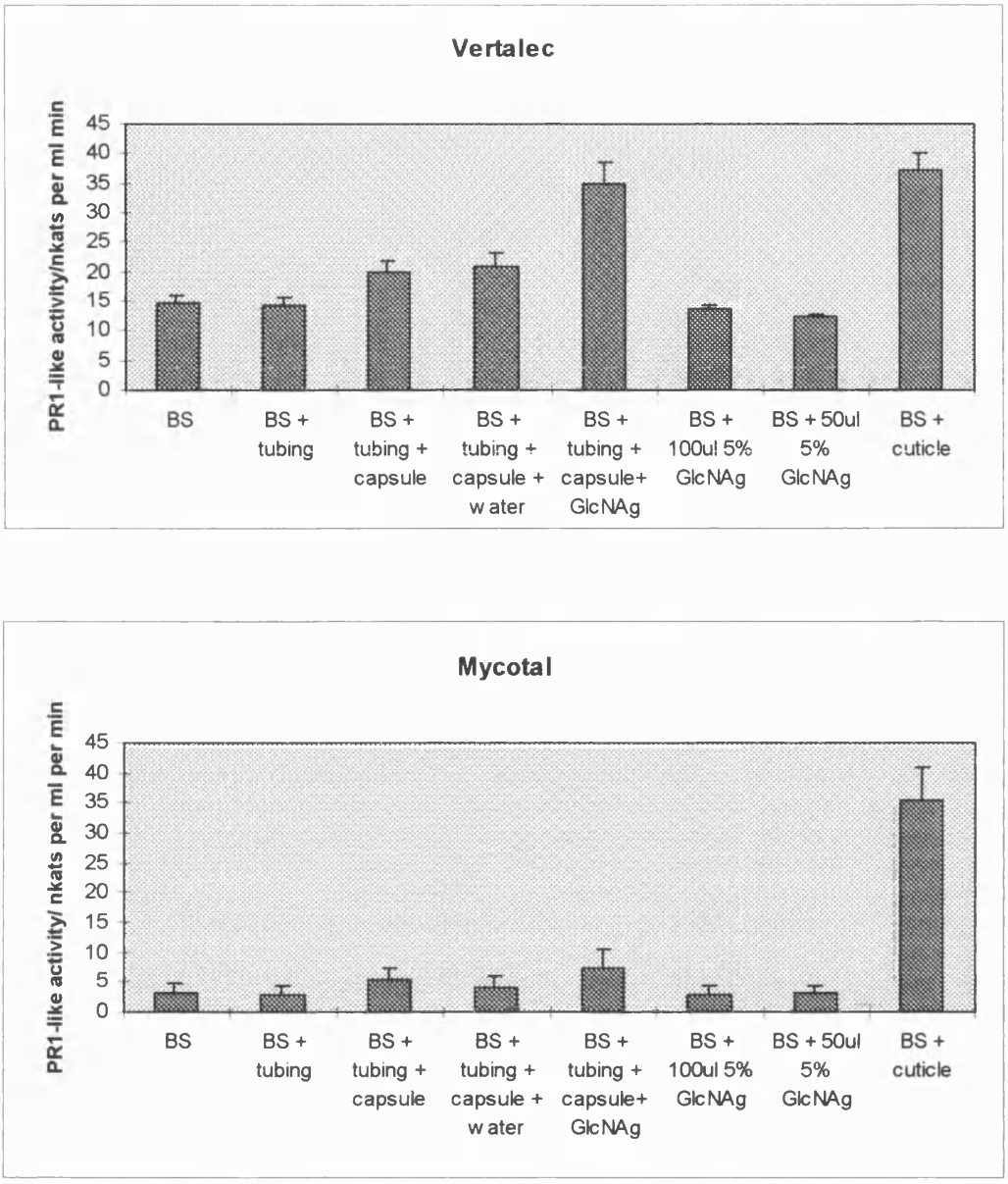


Figure 3.11. The effect of N-acetyl glucosamine on the induction of PR1-like activity 16 hours after transfer into test conditions. Following starvation for 24 hours, 4 day old established biomasses of Vertalec and Mycotal were transferred into the following test conditions and assayed for PR1-like activity 16 hours post transfer. (Activity is expressed as the mean of 4 replicate flasks, standard deviations are shown)

BS	Buffered basal salts medium only
BS + tubing	Basal salts plus one disc of dialysis tubing
BS + capsule	Basal salts plus one diffusion capsule
BS + capsule + water	Basal salts plus one diffusion capsule filled with dH_20 sealed with 1 disc of dialysis tubing
BS + capsule + GlcNAg	Basal salts plus one diffusion capsule filled with 5% N-acetyl glucosamine sealed with 1 disc of dialysis tubing
BS + 100µl 5% GlcNAg	Basal salts supplemented with 100µl of 5% N-acetyl glucosamine
BS + 50µl 5% GlcNAg	Basal salts supplemented with 50µl of 5% N-acetyl glucosamine
BS + cuticle	Basal salts supplemented with 1% w/v locust cuticle

3.1.3.6. A comparison of produced PR1-like activity between two lead isolates of *Verticillium lecanii* on host and non-host cuticle

An established biomass of Vertalec or Mycotal was starved for 24 hours and then transferred into basal salts containing 1% locust or aphid cuticle and PR1-like activity assayed at 16 and 24 hours (figure 3.12.)

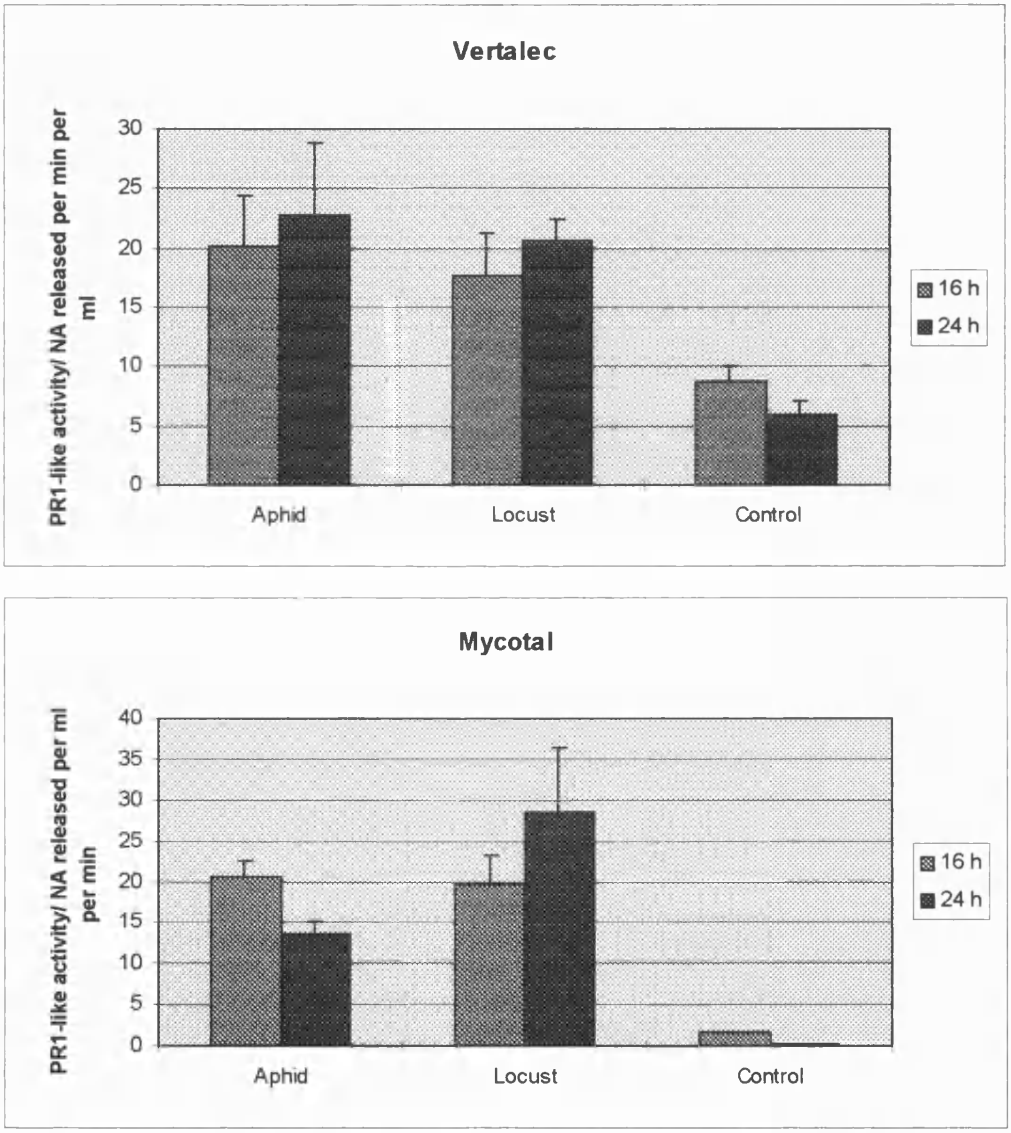


Figure 3.12. PR1-like activity 16 and 24 hours following transfer of a starved biomass of Vertalec and Mycotal into basal salts containing aphid cuticle, locust cuticle or just basal salts(control). Mean activity is expressed as nkats NA released ml^{-1} min^{-1} from four replicate flasks, standard deviations are shown.

In Vertalec cultures, a similar level of PR1-like activity was observed on locust and aphid cuticle, which in both cases, was significantly greater than the control, at both 16 and 24 hours. In Mycotal cultures, at 16 h, again similar PR1-like activity was recorded on both aphid and locust cuticle, however at 24 h, far more protease activity was observed on locust cuticle than on aphid cuticle ($p<0.05$ as determined by t-test).

3.1.3.7 Summary

For all *V.lecanii* isolates studied, substantial PR1-like activity was produced in biomass transfer experiments involving insect cuticle. Protease in cuticle cultures was significantly greater than in basal salt controls, which discounts simple derepression. At least for Vertalec, biomass did not vary between treatments, therefore there was no growth related enzyme synthesis. The results are consistent with regulation by induction. In the case of Vertalec, the inducer appears to be N-acetyl glucosamine, the monomer of chitin, while for Mycotal protein is implicated.

3.1.4 Studies on metabolite repression of PR1-like activity produced by isolates of *Verticillium lecanii*

3.1.4.1 PR1-like activity on cuticle in the presence of additional carbon and nitrogen

The effect of supplementing insect cuticle with more readily available sources of carbon and nitrogen in the form of sucrose (1% w/v) and ammonium chloride (0.4%) respectively was investigated (figure 3.13). As before, statistical comparisons of data were performed using a Oneway Analysis of Variance. At 3,60df, the probability of

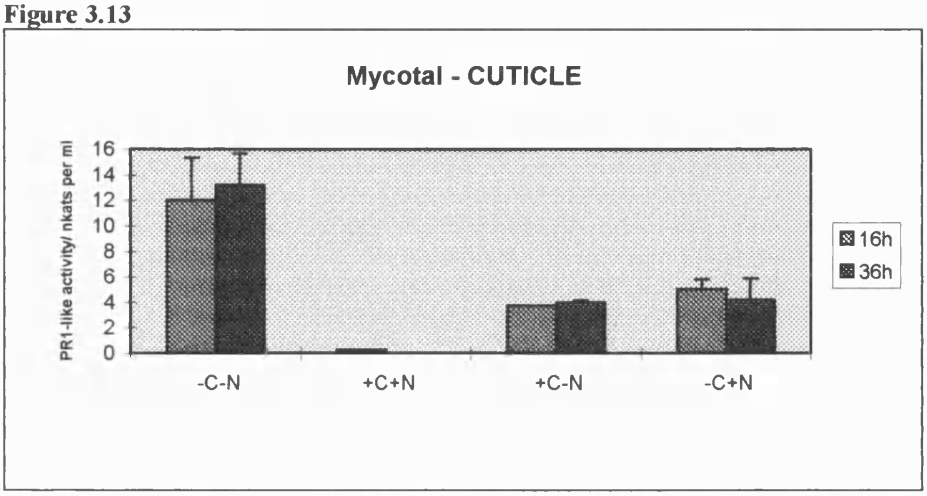
the treatments being the same at 16 or 36 h was found to be less than 0.000 for each isolate.. Fisher's pairwise comparisons highlighted where treatments were significantly different from each other and the actual results of these statistical analyses are shown in the Appendices. (NB: '+' marks any treatments *not* significantly different from cultures containing cuticle as the sole source of carbon and nitrogen).

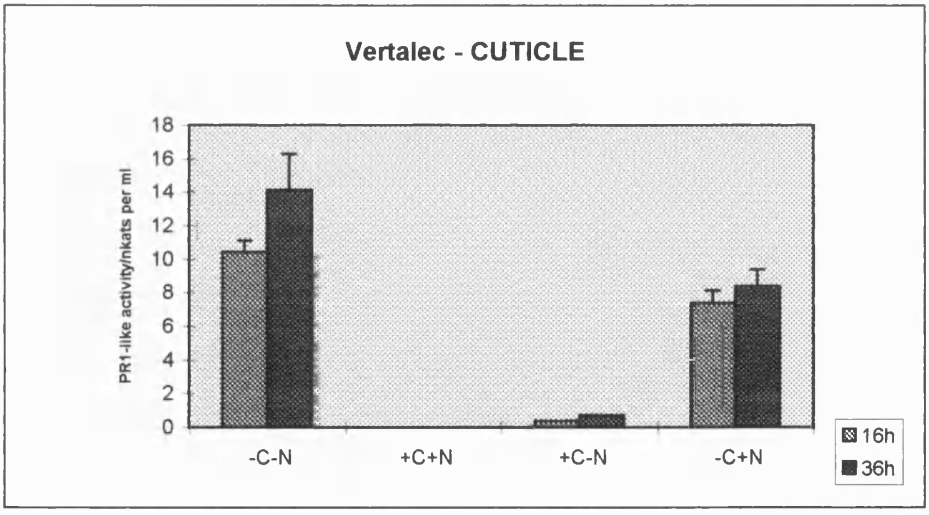
In general, there was no significant difference between PR1-like activity at 16 and 36h, on cuticle as the sole source of carbon and nitrogen. For Vertalec and KV54, significantly less protease activity occurred in cultures containing an additional low molecular weight source of nitrogen. When 1% sucrose was supplied to cuticle cultures, almost total repression of PR1-like activity was observed at both time points. At 16 h, KV22 produced considerably lower levels of PR1-activity in cuticle cultures containing either additional carbon or nitrogen, however at 36h, although activity was repressed in carbon containing cultures, it was significantly enhanced in those cultures containing additional nitrogen. Subtilisin protease production by Mycotal was repressed equally under conditions of additional carbon or nitrogen, at 16 and 36 h.

Strain KV42 at 16 and 36 h did not display any significant differences in PR1-like activity between cultures containing cuticle as the sole source of nutrients, and those containing either additional carbon or nitrogen. In fact when a single additional nutrient source was supplied there appeared to be no repression of the enzyme, unlike for the other strains tested.

KV42, Vertalec, KV22, KV54 and Mycotal, all exhibited almost total repression of PR1-like activity when both soluble nitrogen and carbon were supplied to cuticle cultures at 16 and 36 h; the two nutrients apparently acted synergistically to inhibit enzyme production. (Statistical analyses to confirm statements made can be found in the Appendices).

Figure 3.13





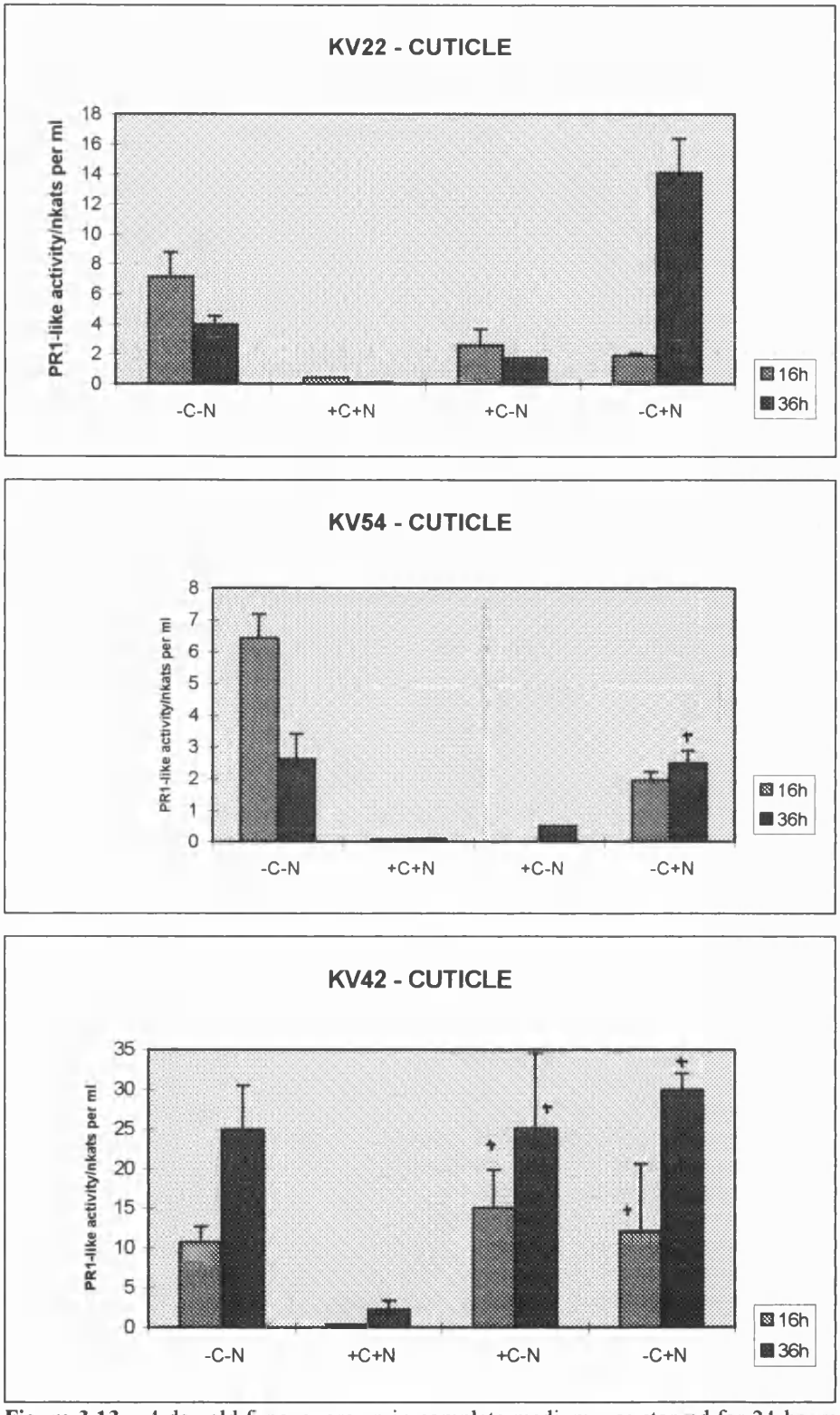


Figure 3.13. 4 day old fungus, grown in complete medium was starved for 24 hours before transfer to cultures containing 1% locust cuticle [-C-N], or 1% locust cuticle in the presence of additional, more readily available forms of carbon and/or nitrogen: [+C+N; +C-N; -C+N]. Enzyme activity against N-Suc-$(Ala)_2$-Pro-Phe-pNA was recorded for each of the five strains shown on these substrates at 16 and 36 h post transfer. Results in nkat NA released min^{-1} ml^{-1} are shown as the mean of 4 replicates. Standard deviations are also shown. [+ = *not* significantly different from -C-N at that time point]

3.1.4.1.1 The use of the fungal sterol 'Ergosterol' as a measure of fungal biomass in repression experiments

As described in section 3.1.3.3, it was necessary to confirm that any differences in PR1-like activity under the conditions tested were not due to changes in biomass of the fungus. Thus the fungal sterol, ergosterol, was again used as a measure of fungal growth. The sterol was extracted from Vertalec cultures, 24 hours after transfer and analysed on HPLC. The biomass of the fungus was then estimated as described previously (figure 3.14.)

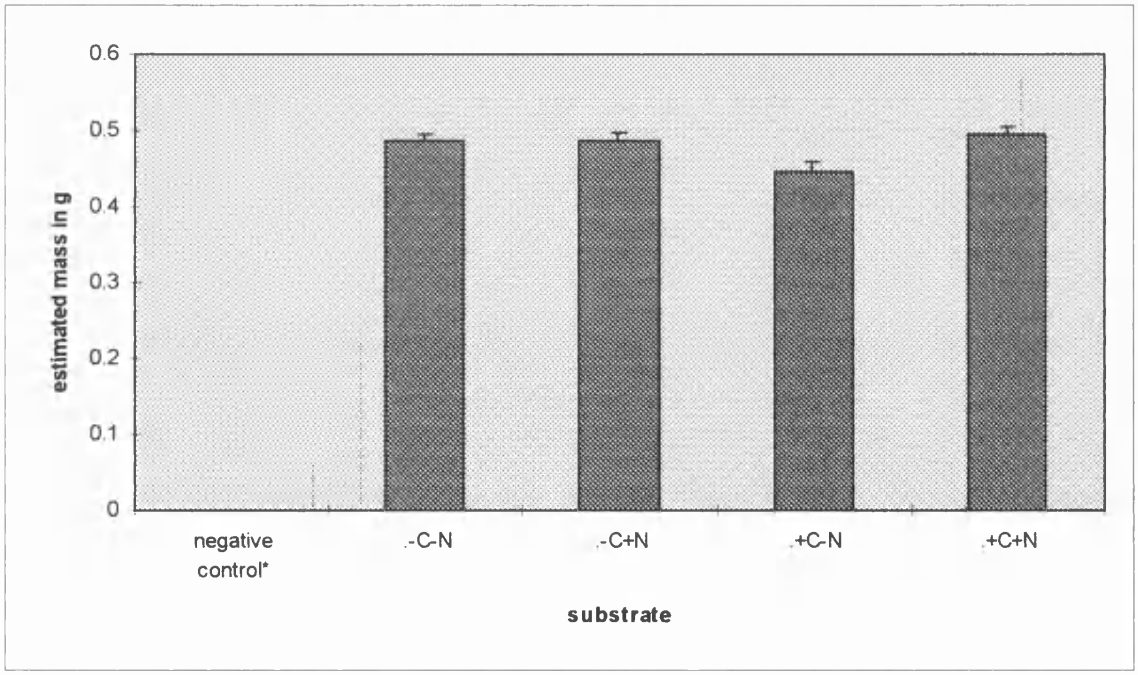


Figure 3.14. Shows the mean estimated biomasses of two replicate extractions of Vertalec from each flask, 24 h post transfer, into cuticle cultures alone (-C-N), or with additional nitrogen, carbon or both (-C+N, +C-N, +C+N). Bars represent the maximal estimated biomass.
[* negative control: no fungal biomass was transferred into substrate flask. Extraction performed on basal salts to ensure no residual sterols were present]

These results indicate that there were no significantly differences in biomass between cultures that could account for differences in protease activity.

3.1.4.2. PR1-like activity in control cultures in the presence of soluble sources of carbon and nitrogen

For 3 of the isolates of *Verticillium lecanii* tested, starvation itself (control cultures) derepressed PR1-like activity, albeit to considerably lower levels than in cuticle cultures (section 3.3.2). The effect of supplementing these cultures of Vertalec, KV22 and KV42 with soluble sources of carbon and nitrogen (1% sucrose and 0.4% ammonium chloride) was investigated (figure 3.15).

For Vertalec, up to 36h PR1-like activity was totally repressed by low molecular weight carbon and partially by low molecular weight nitrogen. Carbon repression was less severe for KV22. For both isolates, addition of soluble carbon and nitrogen completely repressed protease activity. Interestingly, with KV42, although substantial repression occurred in carbon containing cultures, nitrogen had little effect. In combination, soluble carbon and nitrogen substantially suppressed but did not prevent protease production.

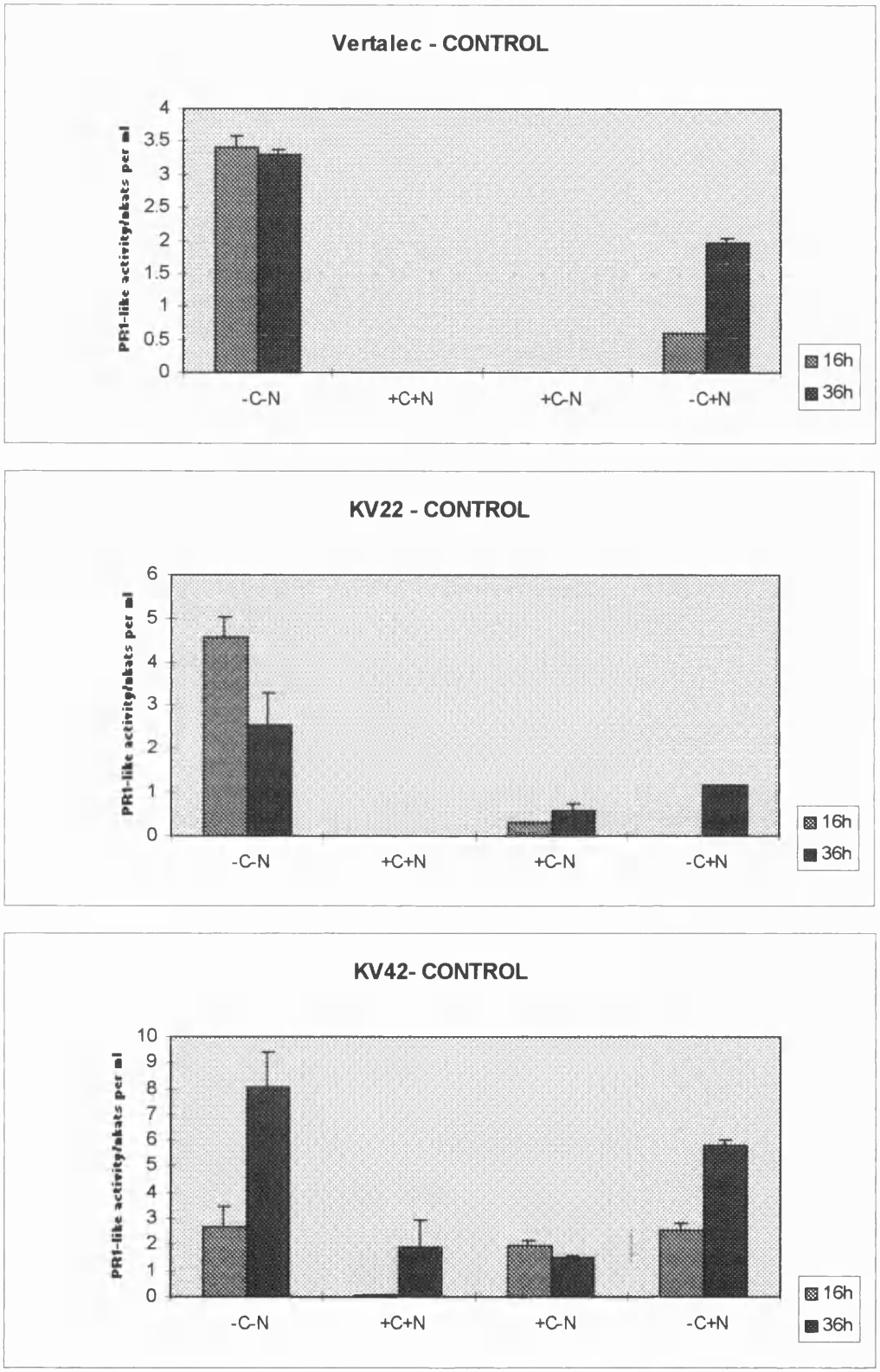


Figure 3.15. 4 day old fungus, grown in complete medium was starved for 24 hours before transfer to basal salts (-C-N), or basal salts supplemented with carbon and/or nitrogen (+C+N, +C-N, -C+N). Enzyme activity against N-Suc-$(Ala)_2$-Pro-Phe-pNA was recorded for each of the five strains shown on these substrates at 16 and 36 h post transfer. Results in nkat NA released min^{-1} ml^{-1} are shown as the mean of 4 replicates. Standard deviations are shown.

3.1.4.3. PR1-like activity in Bovine Serum Albumin cultures in the presence of soluble sources of carbon and nitrogen

For 3 of the isolates of *Verticillium lecanii* tested, PR1-like activity was detected on the soluble protein Bovine Serum Albumin (BSA). The results of supplementing BSA cultures of Vertalec, KV42 and Mycotal with low molecular sources of carbon and nitrogen (1% sucrose and 0.4% ammonium chloride) are shown in figure 3.16.

For KV42 on BSA, as for insect cuticle, addition of either sucrose or ammonium chloride to the medium, did not repress PR1-like activity, indeed a soluble carbon supplement enhanced protease activity. For Vertalec highest levels were found in cultures with no additional carbon and nitrogen. +C, +N and +C+N significantly reduced but did not completely repress PR1-like activity. Finally, for Mycotal, the strain most strongly induced to produce protease on BSA, +C, +N and +C+N all substantially repressed the enzyme. In this experiment, more so than in the others, protease activity in some treatments was significantly less at 36h than 16h, possible because of autolysis.

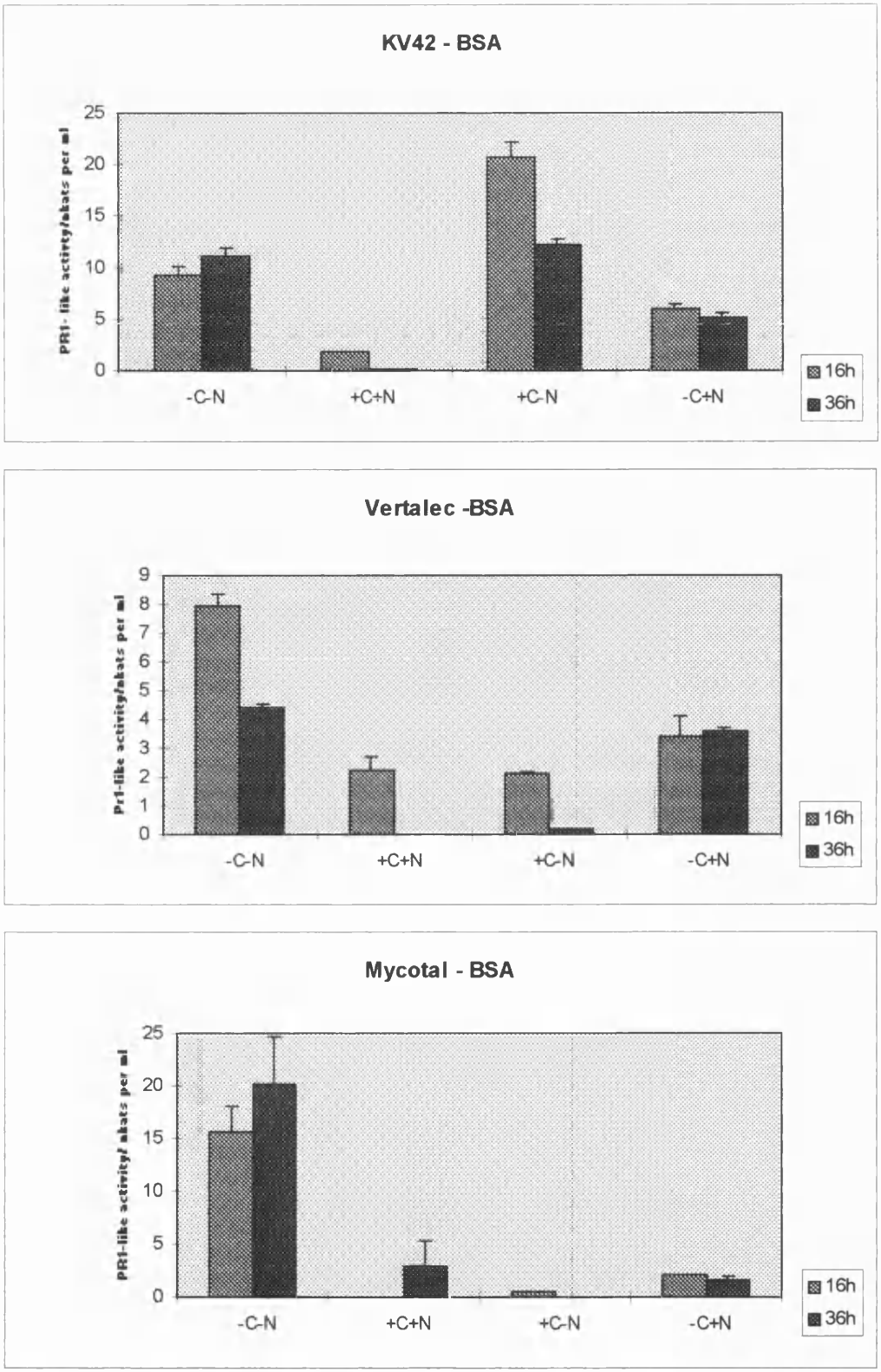


Figure 3.16. 4 day old fungus, grown in complete medium was starved for 24 hours before transfer to basal salts plus BSA (-C-N), or basal salts plus BSA supplemented with carbon and/or nitrogen (+C+N, +C-N, -C+N). Enzyme activity against N-Suc-$(Ala)_2$-Pro-Phe-pNA was recorded for each of the five strains shown on these substrates at 16 and 36 h post transfer. Results in nkat NA released min^{-1} ml^{-1} are shown as the mean of 4 replicates. Standard deviations are shown.

3.1.5 Iso-electric focusing Studies

Regulation of PR1-like enzymes differs between isolates of *V.lecanii*. Vertalec is induced by chitin while Mycotal is under the control of protein. Furthermore, Vertalec is strongly repressed by soluble C and N whereas KV42 is not. The next step was to investigate differences in regulation between isoforms of PR1-like enzymes produced by these 3 isolates.

3.1.5.1 Iso-electric separation of PR1-like enzymes produced by 3 isolates of *Verticillium lecanii* when an established biomass of fungus is transferred to insect cuticle as the sole source of carbon and nitrogen.

a. Coomassie Stain

Broad range (pH 3-10) isoelectric focusing revealed differences in the profile of extracellular proteins in cultures of isolates grown on insect cuticle (Figure 3.17), particularly in the basic area of the gel, where PR1-like activity is anticipated. For example, the couplet of proteins at pI 8.98/8.92 in KV42 is very prominent, is very feint in Mycotal and absent from Vertalec. All isolates consistently produce at least one distinct protein at the limit of the resolution of the gel ($\geq$9.47).

The protein profiles were further investigated for protease activity and subsequently PR1-like activity, to account for different regulation strategies of these key strains.

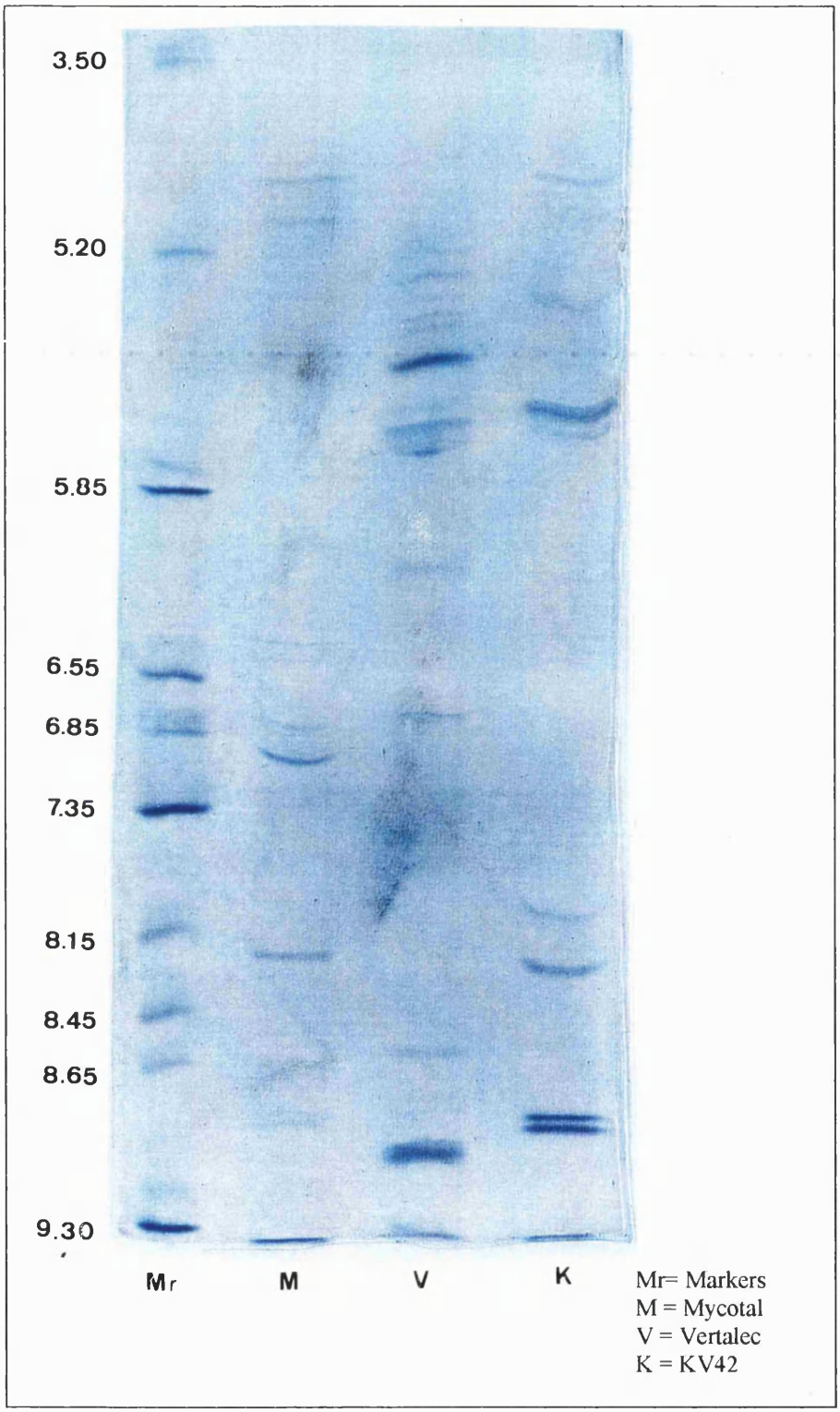


Figure 3.17. Coomassie Stain of proteins from cuticle cultures of *Verticillium lecanii*.
100 µg protein from Mycotal, Vertalec and KV42 cuticle cultures 20 h post transfer was loaded onto a broad range iso-electric focusing gel (Ampholine PAGplate™). Samples were focused at a constant wattage of 30W, fixed and stained with Coomassie blue as per the manufacturer's instructions. Broad range pI markers are shown.

b. Localisation of proteases using a gelatin overlay

Moist x-ray film was used for detecting protease activity. The appearance of degradation zones in the gelatin coat of the overlay film was monitored with time until no further bands were observed. The position at which bands first appeared was noted in order to make an accurate assessment of pI before degradation zones became too diffuse.

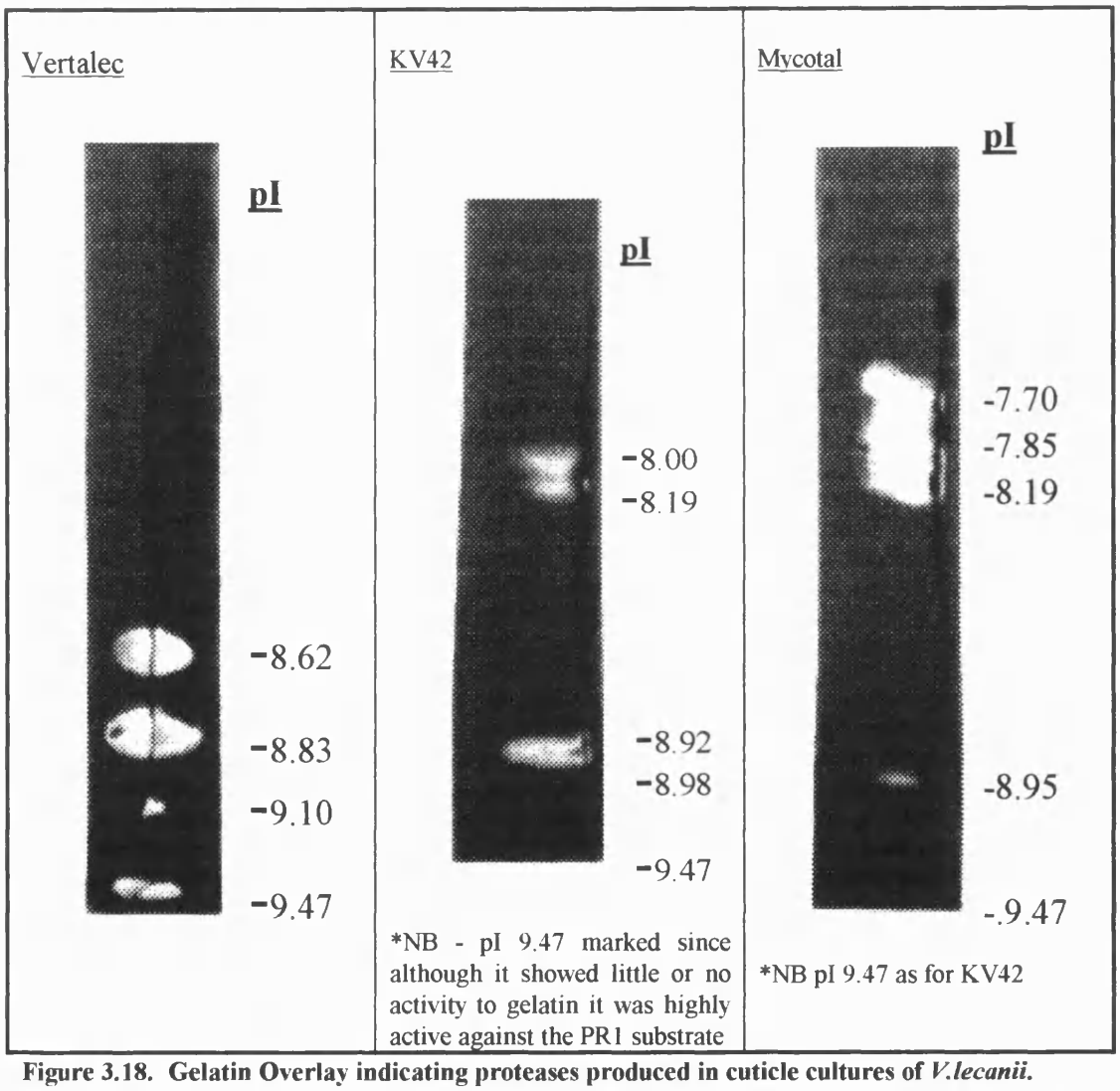


Figure 3.18. Gelatin Overlay indicating proteases produced in cuticle cultures of *V.lecanii*. 50 µg protein from Vertalec, KV42 and Mycotal cuticle cultures, 20 h post transfer, was focused as described previously and overlaid with moist x-ray film. The position of degradation zones were noted and the pI of the protease estimated by comparison to broad range pI markers run on the same gel and stained with Coomassie Blue. The experiment was repeated 3 times with similar results.

Figure 3.18 shows the pattern of protease isoform production by three isolates of *V.lecanii*. Only the basic section of the gel (pH 7-10) is shown since this is the region where chymoelastase activity was detected, (see section 3.1.5.3). Four distinct degradation zones were seen on the gel with Vertalec, indicating proteases with pI values of 8.62, 8.83, 9.10 and $\geq$ 9.47 (the limit of the resolution of the gel). In addition, gelatin degrading activity was also observed at pI 6.48, 6.30 and 5.8 (as shown in section 3.5.2 and 3.5.3). These forms of protease had activity against the PR2-like substrate. KV42 produced 2 very distinct couplets of protease activity at pI 8.00 and 8.19; 8.92 and 8.98. Mycotal appeared to display a different pattern of proteases again, with one gelatin degrading enzyme at pI 8.95 and three forms close to one another at pI 7.7, 7.85 and 8.19. Both Mycotal and KV42 produced high chymoelastase activity at the most basic section of the gel (>9.47), however little or no gelatin degrading activity was observed, compared to that of Vertalec. Mycotal and KV42 also produced a much less basic protease at 6.66 (section 3.5.2 and 3.5.3) which did not have an affinity for the chymoelastase substrate.

c. Localisation of PR1-like enzymes

1mm slices of the gel were taken and homogenised with Tris buffer and chymotrypsin (PR1) substrate, N-Suc-Ala-Ala-Pro-Phe-pNA or trypsin (PR2) substrate, Bz-Phe-Val-Arg-pNA. The release of nitroalanine (NA) was monitored over 1 hour. Figure 3.19 shows the estimated pI for each protease, produced by each of the three strains, as tested against the PR1 and PR2-like substrates.

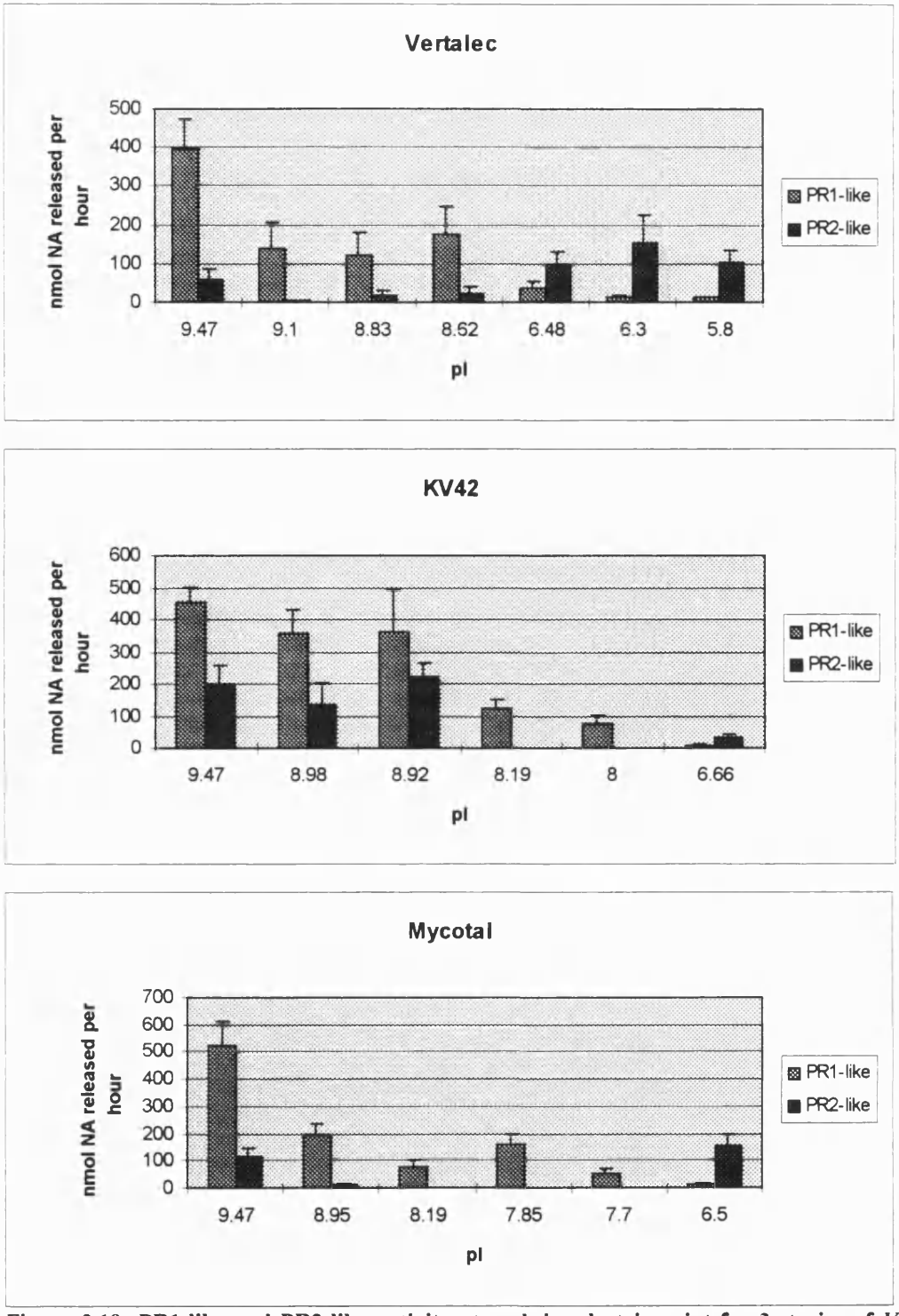


Figure 3.19 PR1-like and PR2-like activity at each iso-electric point for 3 strains of *V.lecanii* grown on insect cuticle. 50µg of protein from each cuticle culture 20 h post transfer was focused on a broad range iso-electric focusing gel as described. 1mm slices were excised from the gel and incubated with N-Suc-Ala-Ala-Pro-Phe-pNA and Bz-Phe-Val-Arg-pNA for PR1 and PR2-like activity respectively. The estimated iso-electric point (pI) where enzyme activity was found is given. The mean activity, in nmol nitroalanine released per hour, and standard deviations from 4 replicates are shown.

Vertalec produced four enzymes that showed activity against the chymoelastase substrate, pI 8.62, 8.83, 9.1 and a highly basic form (≥9.47). The first three showed a poor ability to degrade the PR2-like substrate, though a low level of PR2-like activity was observed at pI 9.47. PR2-like activity appeared to be far higher than that of PR1 for the non-basic forms of protease at pI 6.48, 6.3, and 5.8. Similarly, the main forms of protease detected by gelatin overlay as produced by Mycotal on cuticle, pI ≥9.47, 8.95, 8.19, 7.85, and 7.7 all showed activity against the chymoelastase substrate and, with the exception of pI 9.47, PR2-like activity was not observed for these forms. The PR1-like activity of this very basic form, which did not degrade gelatin, was approximately 10 fold higher than that of pI 7.7 and 8.19, which were not highly active against the chymoelastase substrate. PR2-like activity was detected in a protease with a pI of approximately 6.5, this form did not degrade the PR1-like substrate.

The five main proteases of KV42, detected by gelatin overlay, at pI 8.0, 8.19, 8.92, 8.98, and >9.47 all hydrolysed the PR1-like substrate, the latter three being highly active at degrading it. The protease observed at pI 6.66 showed only a poor activity against the PR2-like substrate and no activity against the PR1-like substrate. In contrast to Mycotal and Vertalec, the three most basic forms of chymoelastase enzyme produced by KV42, also showed a strong activity against the PR2-like substrate. No PR2-like activity was observed by proteases at pI 8.19 and 8.0.

d. The use of inhibitors to characterise the isoforms

To further reveal the nature of the proteases, gel slices were incubated with one of four inhibitors at the recommended concentration (Figure 3.20). Residual activity was then

expressed as a proportion of that activity in the absence of inhibitor (100%), with the appropriate amount of solvent that that inhibitor was dissolved in as a negative control

a. Mycotal

	% of max. activity		
Isoform	Chymostatin	Leupeptin	Turkey Egg White
>9.47	2.4	99.0	7.6
8.95	19.2	84.3	4.3
8.19	29.9	75.4	94.5
7.85	11.9	55.2	110.0
7.70	18.5	44.4	88.9

b. Vertalec

	% of max.activity		
Isoform	Chymostatin	Leupeptin	Turkey Egg White
>9.47	3.03	45.2	18.9
9.10	8.5	87.3	59.4
8.83	30.3	106.0	3.65
8.62	31.5	89	21.4

c. KV42

	% of max. activity		
Isoform	Chymostatin	Leupeptin	Turkey Egg White
>9.47	0.9	77.0	4.4
8.98	30.2	93.2	0.7
8.92	46.5	94.4	0.8
8.19	38.1	76.2	105.0
8.00	17.3	41.8	109.0

Figure 3.20 The effect of the inhibitors chymostatin, leupeptin and turkey egg white on the PR1-like activity of different proteases produced by each strain. Gel slices of the appropriate pI were excised and incubated with water, DMSO (negative controls) or chymostatin, leupeptin or turkey egg white, at concentrations of 100μM, 100μm and 0.1% respectively. Activity is shown as a percentage of that without inhibitor. (The experiment was repeated 3 times, see figure 3.19 for actual values and standard deviations for 100% activity, DMSO did not inhibit PR1-like activity).

Chymostatin (Phe-(Cap)-Leu-Phe-al) inhibits chymotrypsin like serine proteases and most cysteine proteases. At a concentration of 100μM, in this study all putative PR1-

like enzymes produced by these three isolates of *V.lecanii* showed strong inhibition by chymostatin. For the most basic form of the enzyme ($\geq$ 9.47) for all isolates, there was virtually no activity after incubation with chymostatin, and for isoforms of pI 9.10 and pI 7.85 of Vertalec and Mycotal respectively, activity was reduced to less than 10% suggesting the properties of these enzymes as chymotrypsins or cysteine proteases. The other forms of enzymes produced by Mycotal, Vertalec and KV42 were inhibited by chymostatin to between approximately 20 and 30%, again suggesting that they shared similarities with chymotrypsin or cysteine proteases. The isoform pI 8.92 produced by KV42 was least affected by chymostatin at this concentration, after incubation maintaining almost 50% maximum activity.

Leupeptin (N-Acetyl-Leu-Leu-Arg-Al) is an inhibitor of trypsin-like serine proteases and most cysteine proteases. After incubation with Leupeptin, (100μM), the majority of proteases tested maintained between 70 and 99% activity against the subtilisin substrate, suggesting that these forms are chymotrypsins and not cysteine proteases as indicated by chymostatin. Total inhibition was not observed for any protease, however, for 2 isoforms of Mycotal, (pI 7.85 and 7.7), one isoform of Vertalec (pI $\geq$9.47) and one isoform of KV42 (pI 8.0), leupeptin inhibited activity to approximately 50% of the maximum. This would indicate that these forms, inhibited by chymostatin as well, may share some properties of a cysteine protease or trypsin-like serine protease.

Finally, turkey egg white inhibitor, and inhibitor of chymotrypsin and trypsin, showed marked differences in its effects on the proteases. For KV42 and Mycotal, the most

basic proteases (>9.47, 8.98 and 8.92) and (>9.47 and 8.95) activity against the PR1-like substrate was almost completely repressed following incubation with turkey egg white (at 0.1%w/v), supporting the idea that these proteases are chymotrypsins/trypsins. However, for the less basic forms of protease from KV42 and Mycotal (8.19 and 8.0) and (pI 8.19, 7.85, 7.7) respectively, no effect by turkey egg white was observed on their ability to hydrolyse N-Suc-Ala-Ala-Pro-Phe-pNA. The isoforms of Vertalec all showed a level of inhibition by turkey egg white, although one form (pI 9.10) still maintained activity of almost 60% of that of the maximum.

NOTE: For those isoforms that also showed a level of activity against the PR2-like substrate, the experiment was repeated using this substrate(Bz-Phe-Val-Arg-pNA). The pattern of inhibition (not shown) was almost identical to that shown in figure 3.20, suggesting that a single enzyme was present and not two in each case.

Finally, 1, 10 phenanthroline, a known inhibitor of metalloproteases, was tested against the PR1-like enzymes, however, no inhibition was observed at 2mM on any form of the enzyme from each of the three isolates.

3.1.5.2 Iso-electric separation of PR1-like enzymes produced by Vertalec and KV42 when an established biomass of fungus is transferred to insect cuticle with or without additional sources of carbon and nitrogen.

Aliquots of concentrated culture filtrate, containing 50 µg protein, from Vertalec and KV42 grown on one of cuticle alone (-C-N) cuticle plus soluble nitrogen (-C+N), cuticle plus soluble carbon (+C-N) or cuticle plus soluble carbon and nitrogen (+C+N). were loaded onto an iso-electric focusing gel as described previously. Differences were noted in gelatin overlays and gel slice assays.

a. Gelatin Overlay - Vertalec

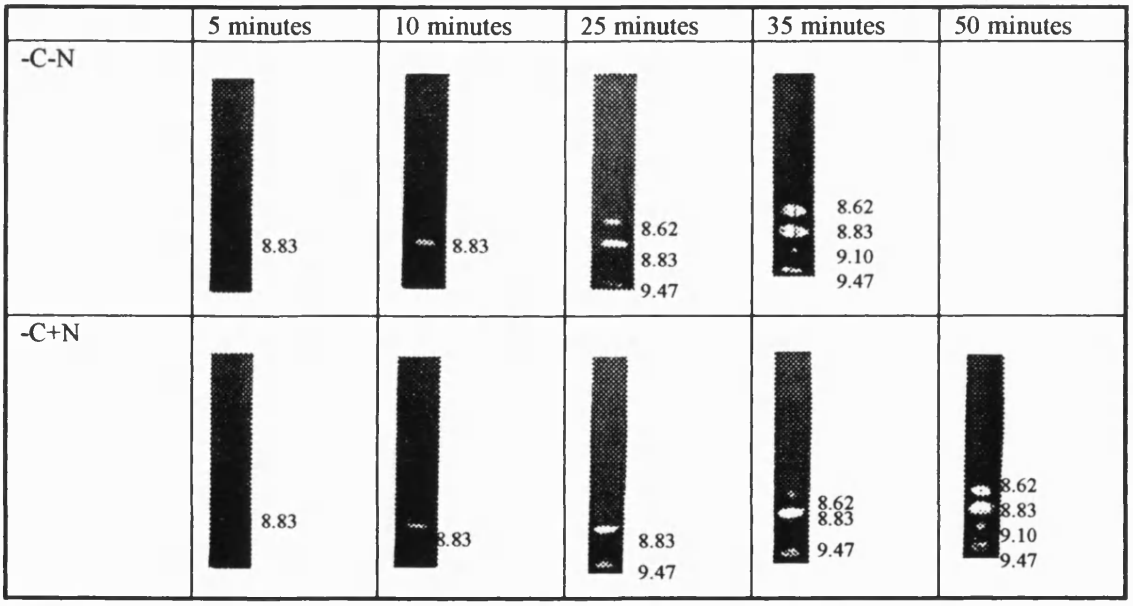


Figure 3.21 Degradation of gelatin overlay with time. Focused lanes of the gel were overlaid with moist x-ray film and degradation zones were noted and photographed as they developed, key time points are shown for cuticle alone (-C-N) and cuticle with additional nitrogen (-C+N). No activity against the gelatin overlay was detected for cuticle with additional carbon (+C-N) or carbon and nitrogen (+C+N). Similar results were found when the experiment was repeated with three replicate samples.

No degradation zones were observed in lanes of the gel containing samples from cuticle cultures where additional carbon had been supplied. Four distinct degradation

zones were observed for the Vertalec sample from culture containing cuticle as the sole source of carbon and nitrogen as observed earlier. The isoform with pI 8.83 appeared to be first to degrade the gelatin, followed by that of 8.62, then the most basic form, >9.47. The last form to degrade the gelatin was the isoform of pI9.10. This protease appeared to be reduced in cultures supplemented with nitrogen. In addition, the protease of pI 8.62 was also slower to degrade the gelatin in soluble nitrogen containing cultures. None of the forms were completely repressed by the inclusion of nitrogen in the medium.

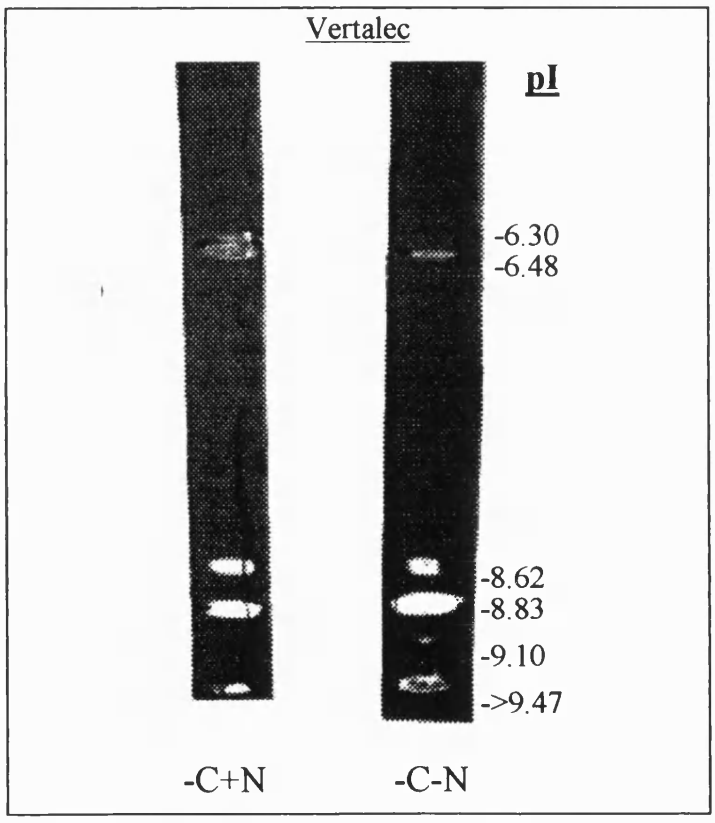
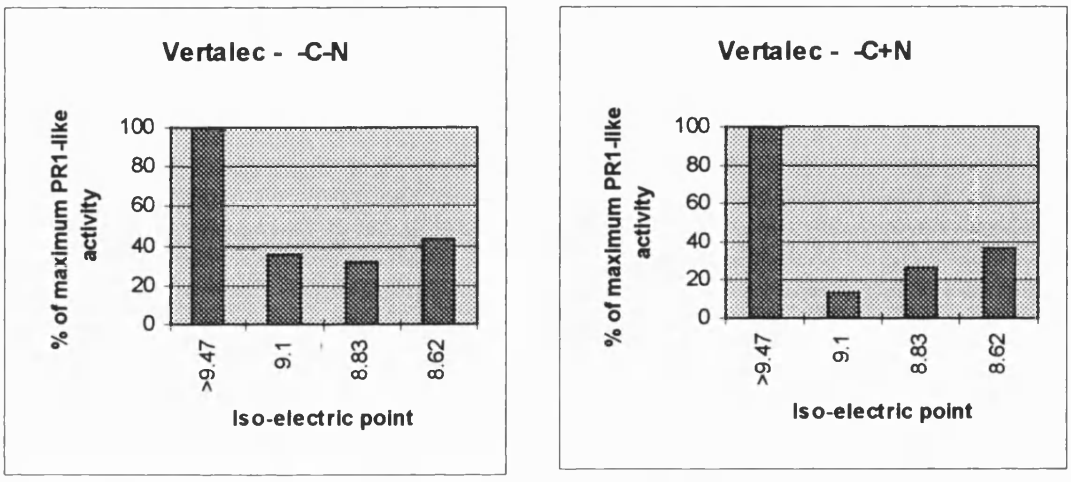


Figure 3.22 Summary of gelatin overlay for Vertalec.

b. PR1-like activity - Vertalec

1mm slices were excised from the gel at each pI measured and incubated with the substrate N-Suc-Ala-Ala-Pro-Phe-pNA, the release of nitroalanine (NA) was monitored over 1 hour. Figure 3.23 shows PR1-like activity as a percentage of the highest producer (pI >9.47), recorded for sample lanes where activity was detected (cuticle -C-N and cuticle -C+N). Actual maximum activity and standard deviation is shown.



100% = 398.02 ± 75.7 nmol NA h^{-1} 100% = 317.11 ± 102.7 nmol NA h^{-1}

Figure 3.23. Isoforms of a PR1-like enzyme produced by Vertalec under different carbon and nitrogen nutrient regimes. Activity of the excised gel slices against the chymotrypsin substrate is expressed as a percentage of the highest activity (100%) recorded for each sample lane. The actual release of nitroalanine per hour and standard deviation are shown for 100% as a means for comparison (n=3).

Although not the fastest to degrade gelatin, in both cuticle cultures with or without additional nitrogen, the most basic isoform, pI ≥9.47 was the most active against the subtilisin substrate. In cuticle-only cultures, the other three isoforms, 9.1, 8.83 and 8.62 hydrolysed the substrate to a rate of approximately 40% of that of pI 9.47. The protease with pI 8.83 was the slowest to degrade this substrate, yet it was the first to degrade the gelatin overlay. Consistent with the findings of the overlay, however, the isoform with pI 9.10 in cultures containing additional nitrogen, exhibited a four fold

reduction in activity against N-Suc-Ala-Ala-Pro-Phe-pNA compared to that found in cultures containing cuticle alone.

c. Gelatin Overlay - KV42

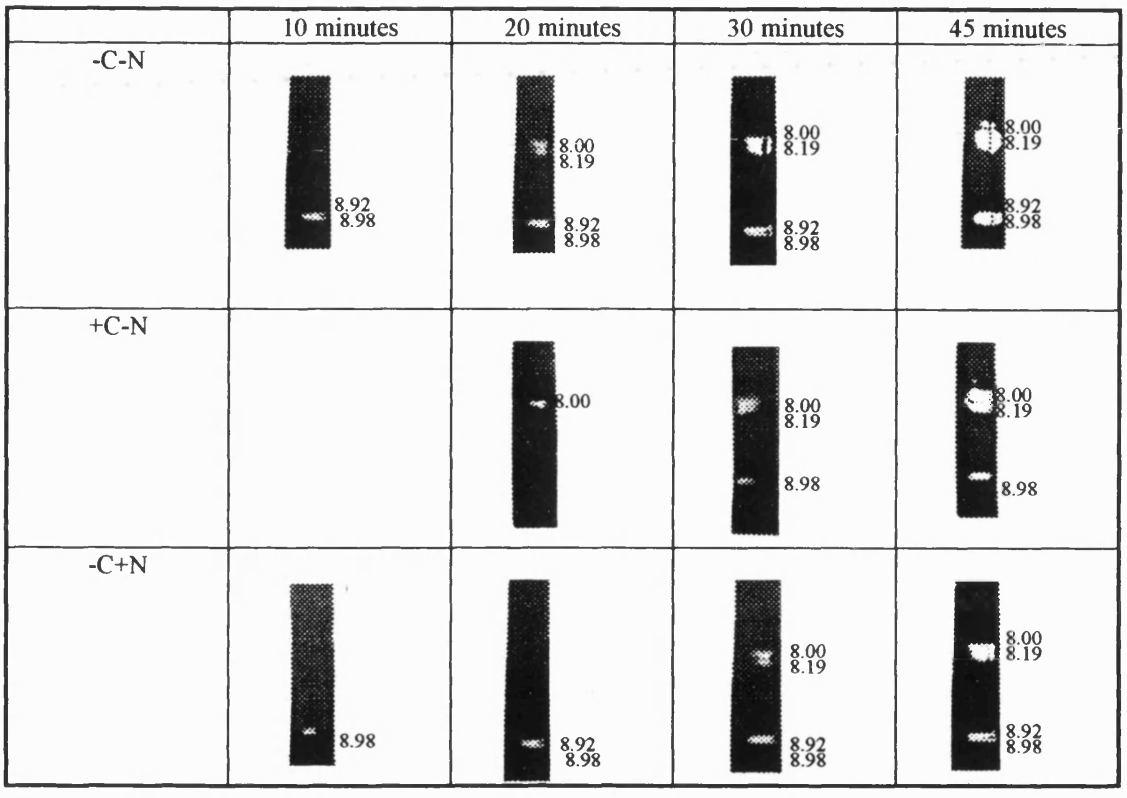


Figure 3.24 Degradation of gelatin overlay with time. Focused lanes of the gel were overlaid with moist x-ray film and degradation zones were noted and photographed as they developed, key time points are shown for cuticle alone (-C-N), cuticle with additional carbon (+C-N) and cuticle with additional nitrogen (-C+N). No activity against the gelatin overlay was detected for cuticle with additional carbon *and* nitrogen (+C+N). Similar results were found when the experiment was repeated with three replicate samples.

The pattern of gelatin degradation by culture filtrate from cuticle cultures with or without additional nitrogen was virtually indistinguishable. The first to appear was the couplet of isoforms with pI values of 8.92 and 8.98. These could only be distinguished until 30 minutes, after which the two zones merged into one. The couplet at pI 8.00 and 8.19 was next to form. No degradation was observed at the most basic end of the

gel (≥9.47) where PR1-like activity had been detected, even after leaving the overlay for a further 2 hours. For the proteases of carbon supplemented cultures, the pattern of gelatin degradation appeared to be reversed. The couplet at 8.0 and 8.19 was first to be detected on the overlay. Only at 30 minutes did the enzyme with estimated pI 8.98 begin to degrade the gelatin. However, the second enzyme (pI 8.92) was not clearly distinguishable, the overlay was monitored further over time, however it appeared that the degradation zone became more diffuse rather than a new one appearing.

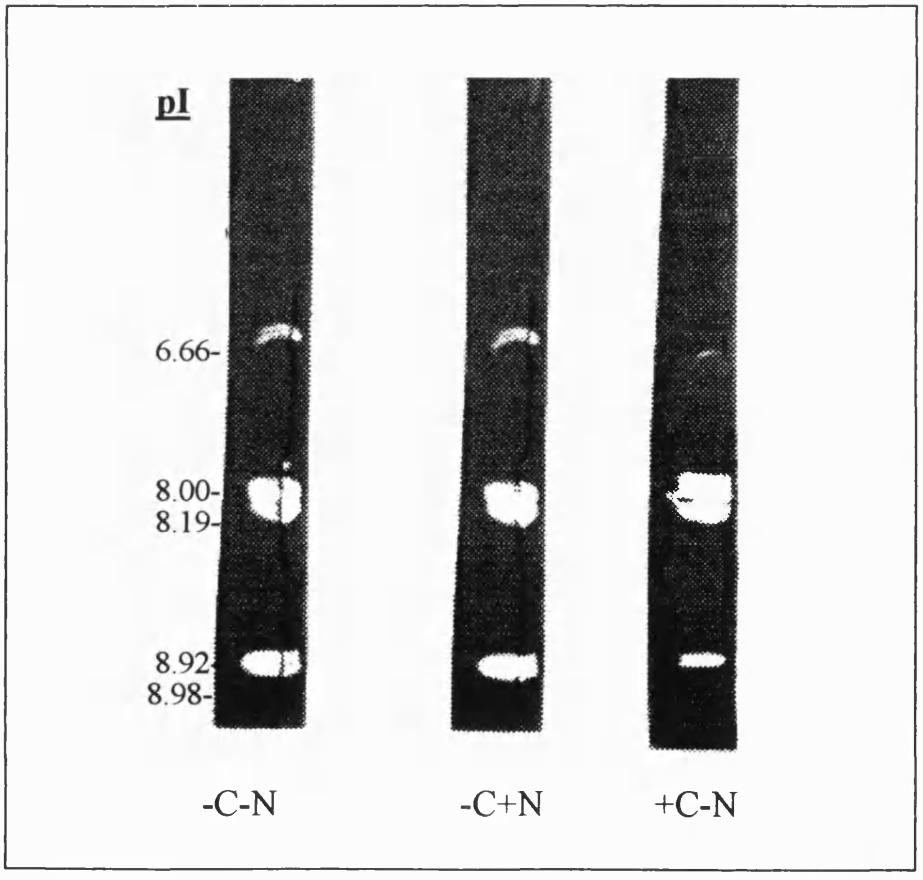
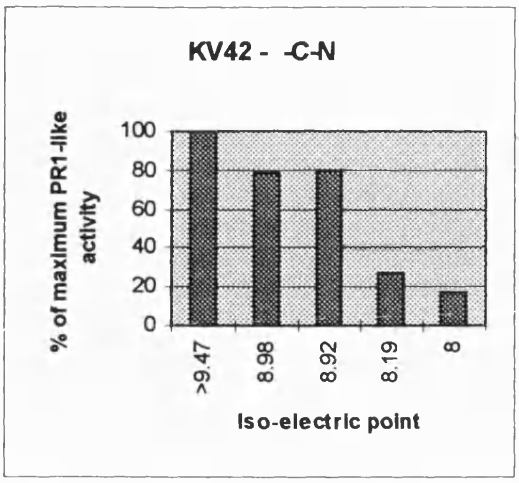
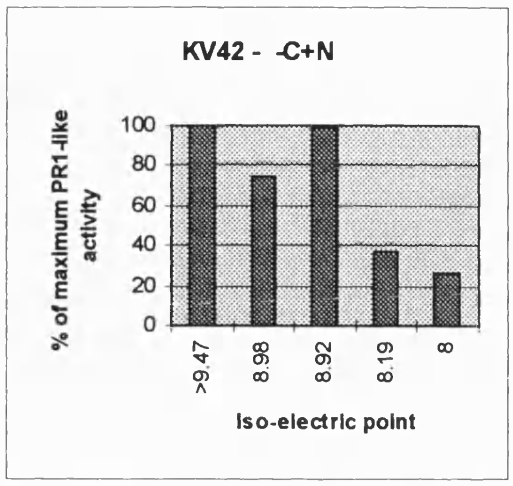


Figure 3.25 Summary of gelatin overlay for KV42.

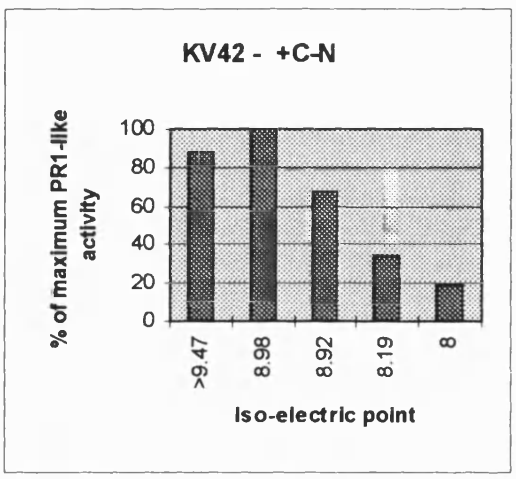
d. PR1-like activity - KV42





100% = 456.68 ± 45.0 nmol NA h^{-1}

100% = 434.08 ± 11.7 nmol NA h^{-1}



100% = 309.35 ± 45.38 nmol NA h^{-1}

Figure 3.26. Isoforms of a PR1-like enzyme produced by KV42 under different carbon and nitrogen nutrient regimes. Activity of the excised gel slices against the chymotrypsin substrate is expressed as a percentage of the highest activity (100%) recorded for each sample lane. The actual release of nitroalanine per hour and standard deviation are shown for 100% as a means for comparison (n=3).

For each of the three sets of culture conditions for KV42, it appears that three isoforms of protease, pI $\geq$ 9.47, 8.98 and 8.92 were the most active against the chymoelastase substrate. The actual maximum activity shown in conditions of cuticle alone or with additional nitrogen were comparable, however when additional carbon was supplied, the PR1-like activity was slightly reduced. No activity was observed when carbon and nitrogen was supplied. The gelatin overlay did not indicate that

isoform pI 8.92 was present, in carbon containing cultures, however high levels of PR1-like activity were still recorded in this gel slice, which was immediately excised from the gel to prevent diffusion of the protease at pI 8.98. In each case isoform pI 8.19 hydrolysed the substrate to between 20 and 40% of that of the highest activity, and pI 8.0 was only 20% active. The patterns of isoform production observed were very similar between the three test conditions, suggesting that additional carbon or nitrogen did not affect the production of the isozymes produced.

When grown on insect cuticle as the sole source of carbon and nitrogen, Vertalec appears to produce 4 main proteases which demonstrate activity against the chymoelastase substrate used in this study. When nitrogen is added to these cultures, one form of the enzyme, (pI 9.10) although still present, is significantly repressed. In the presence of carbon or carbon and nitrogen no proteases with PR1-like activity were detected. KV42 produced a very basic ($\geq$9.47) form with comparable activities to that produced by Vertalec. However, it also produced four other forms of the enzyme with different pI values to that of Vertalec. These forms did not appear to be repressed in the absence of carbon or nitrogen, but no PR1-like activity was observed when the two were supplied together.

Summary

Vertalec

Estimated pI			
-C-N	-C+N	+C-N	+C+N
≥ 9.47 9.10 8.83 8.62	≥ 9.47 (9.10) ** 8.83 8.62	No PR1-like activity detected in lane	No PR1-like activity detected in lane

** isoform 9.10 was slower to degrade the gelatin overlay, and exhibited a 4 fold reduction in PR1-like activity compared to the form produced in the absence of additional nitrogen.

KV42

Estimated pI			
-C-N	-C+N	+C-N	+C+N
≥ 9.47 8.98 8.92 8.19 8.00	≥ 9.47 8.98 8.92 8.19 8.00	≥ 9.47 8.98 8.92 8.19 8.00	No PR1-like activity detected in lane

3.1.5.3. Iso-electric separation of PR1-like enzymes produced by Vertalec and Mycotal when an established biomass of fungus is transferred to different sources of carbon and nitrogen.

Aliquots of concentrated filtrates containing 50 μg protein from cultures of Vertalec and Mycotal on insect cuticle, chitin, KOH treated chitin (to remove all traces of protein) Bovine Serum Albumin (BSA) or basal salts (control) were loaded onto a broad range iso-electric focusing gel. Gelatin overlays and gel slice assays were performed as described previously.

a. Gelatin Overlay - Vertalec

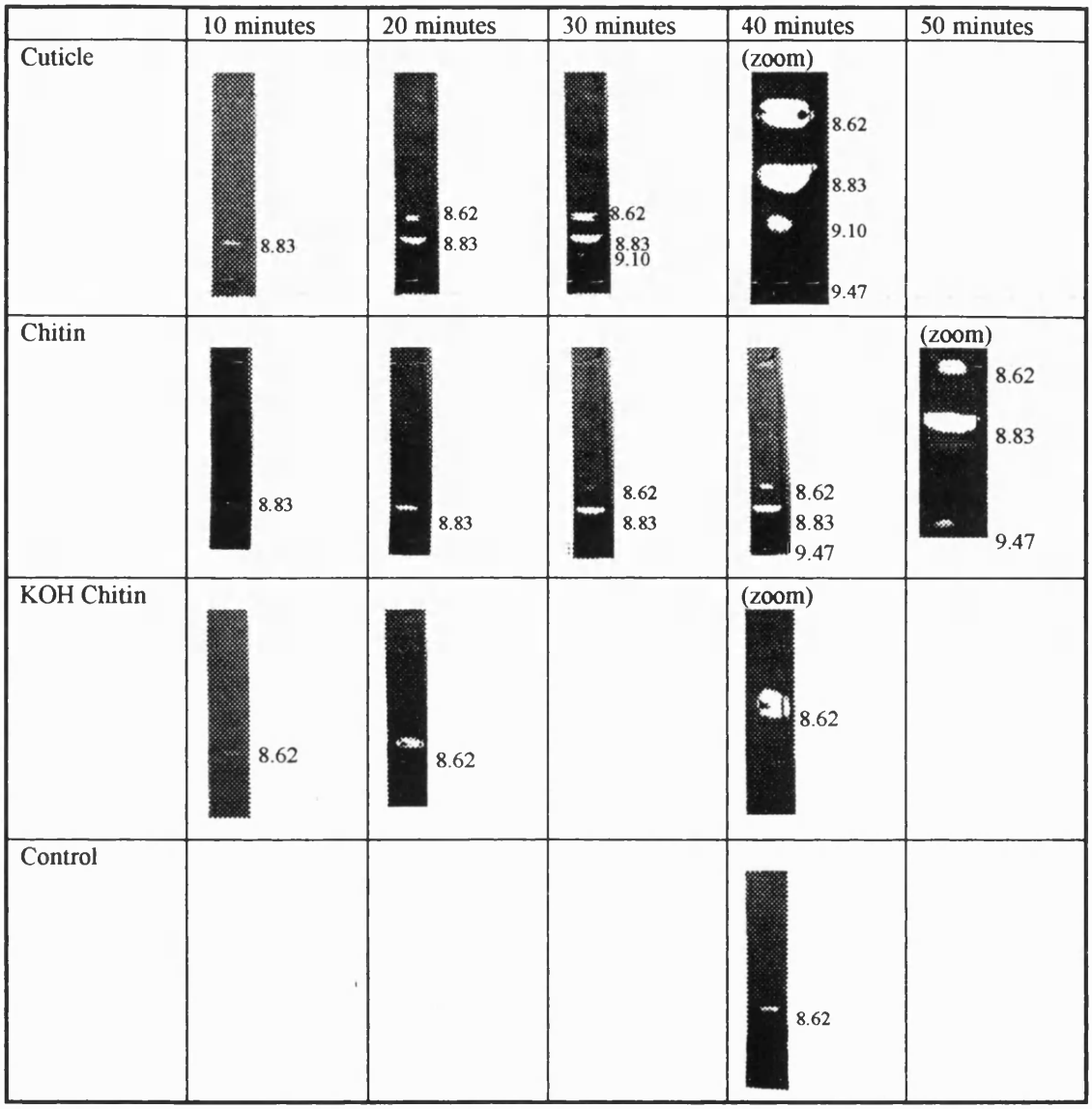


Figure 3.27. Degradation of overlay with time. Focused lanes of the gel were overlaid with moist x-ray film and degradation zones were noted and photographed as they developed. Key time points are shown for locust cuticle, practical grade chitin (from crab shells), deproteinised chitin (KOH treated) and control - basal salts alone. No activity against the gelatin overlay was observed for Bovine Serum Albumin (BSA). Similar results were found when the experiment was repeated with three replicate samples.

As shown in figure 3.21 previously, Vertalec biomass transferred to cuticle, produced four distinct degradation zones in the basic area of the gelatin overlay, (degradation was also observed at pI 6.48, where PR2-like activity had been previously detected). The ability of the most basic form ($\geq$ 9.47) to degrade gelatin appears to be variable.

In this instance, the appearance of forms pI 8.83 and 8.62 was as previously found, but isoform 9.10 appeared before that of 9.47, which was slow to degrade the overlay. The pattern of protease production in cultures of practical grade chitin was very similar to that of cuticle, however no band was observed at pI 9.10. In cultures of chitin, treated to remove protein (KOH), only one form could be seen to degrade the overlay, at pI 8.62. In control cultures, no signs of degradation were observed until 40 minutes after applying the overlay, again it was the appearance at pI 8.62. PR1-like activity in cultures containing Vertalec transferred into Bovine Serum Albumin, were lower than found in original regulation experiments, and when applied to an Iso-electric focusing gel, no activity against the gelatin was observed.

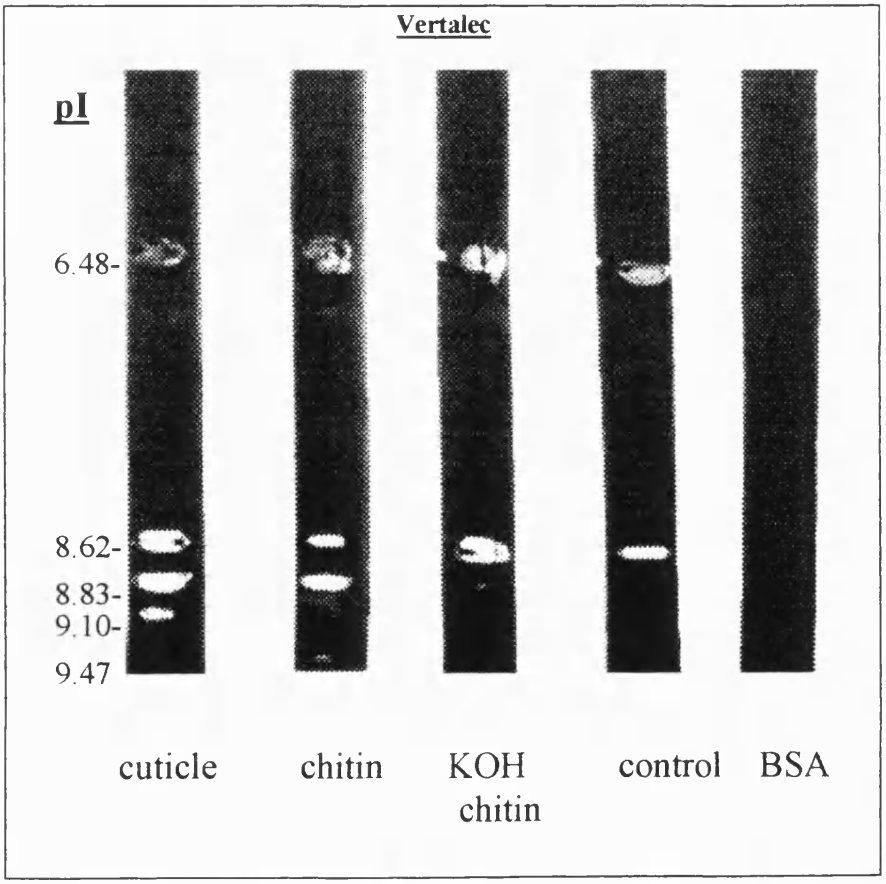
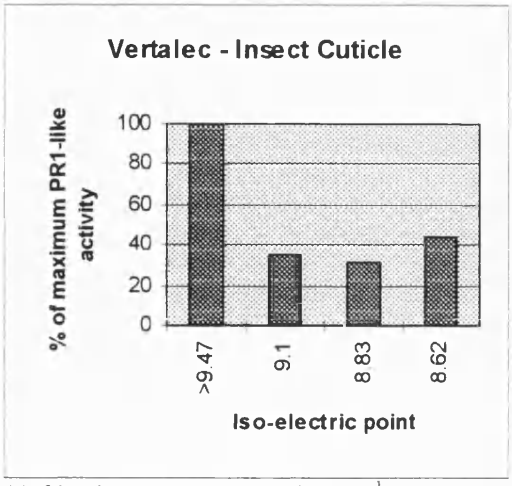


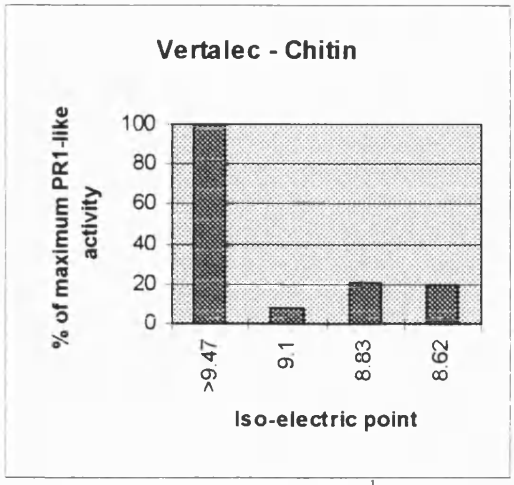
Figure 3.28. Summary of gelatin overlay for Vertalec

b. PR1-like activity - Vertalec

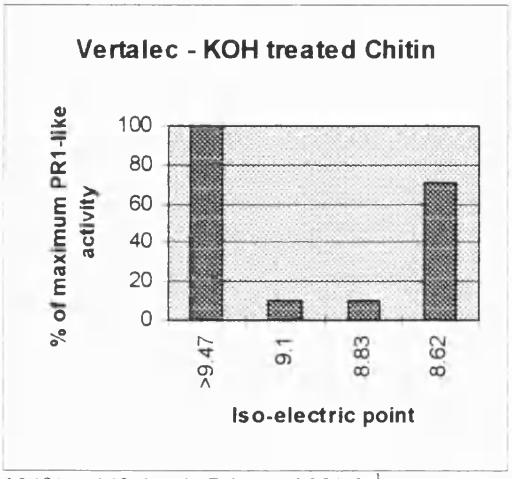
In all substrate conditions, it would appear that the highest PR1-like activity was observed within the most basic slice of the gel (pI $\geq$9.47). In Bovine Serum Albumin (BSA) containing cultures it would appear that only this isoform of PR1-like enzyme was present, albeit in lower levels than other conditions. In cultures containing locust cuticle, as found previously four distinct isozymes, with pI values of $\geq$9.47, 8.62, 9.10 and 8.83 were found (in descending levels of chymotrypsin activity). In cultures containing practical grade chitin from crab shells, the production of the latter three forms was lower than in cuticle cultures, and isoform pI 9.1 was repressed. In cultures of deproteinised chitin (KOH chitin) although isoforms 9.1 and 8.83 appeared to be repressed, production of the protease at pI 8.62 was significantly enhanced compared to that in cuticle cultures. A similar pattern was also observed in control cultures, which had developed much higher levels of PR1-like activity than were observed in original regulation experiments.



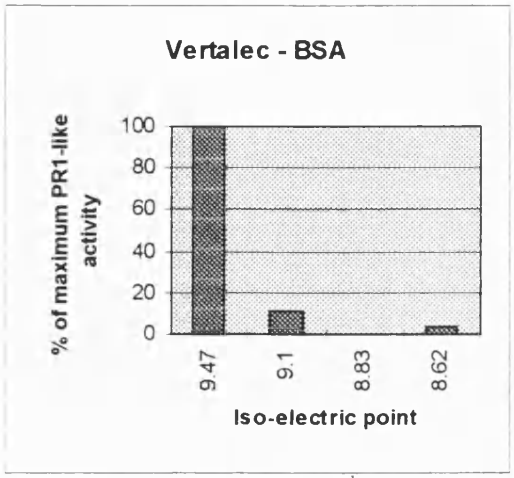
100% = 398.02 ± 75.7 nmol NA h^{-1}



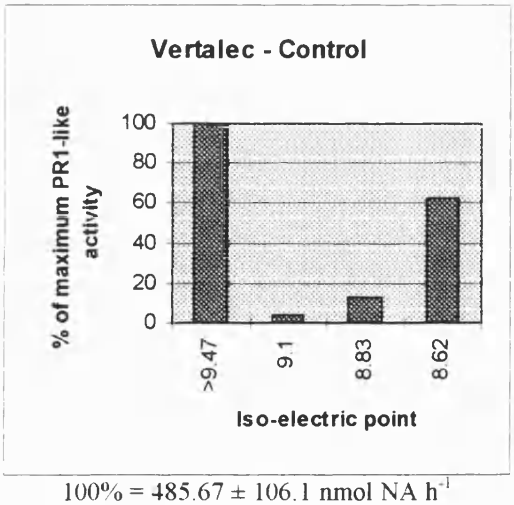
100%= 399.03 ± 99.3 nmol NA h^{-1}



100% = 462.4 ± 107.8 nmol NA h^{-1}



100% = 50.10 ± 8.0 nmol NA h^{-1}



100% = 485.67 ± 106.1 nmol NA h^{-1}

Figure 3.29. Isoforms of a PR1-like enzyme produced by Vertalec on different induction conditions. Activity of the excised gel slices against the chymotrypsin substrate is expressed as a percentage of the highest activity (100%) recorded for each sample lane. The actual release of nitroalanine per hour and standard deviation are shown for 100% as a means for comparison (n=3).

c. Gelatin Overlay - Mycotal

In cuticle containing cultures of Mycotal, the first isoform to degrade the gelatin was that of pI 7.85, which was subsequently accompanied by the isoforms of pI 7.7 and 8.19. Protease activity at pI 8.95 was last to develop. In chitin containing cultures, degradation of the overlay was much slower, and at 40 minutes, isoforms of pI 7.7 and 7.85 could be observed. In cultures where the crab shell chitin had been treated with potassium hydroxide to remove all trace protein, only one form at pI 8.19 was detected.

	10 minutes	25 minutes	30 minutes	40 minutes
Cuticle				
Chitin				

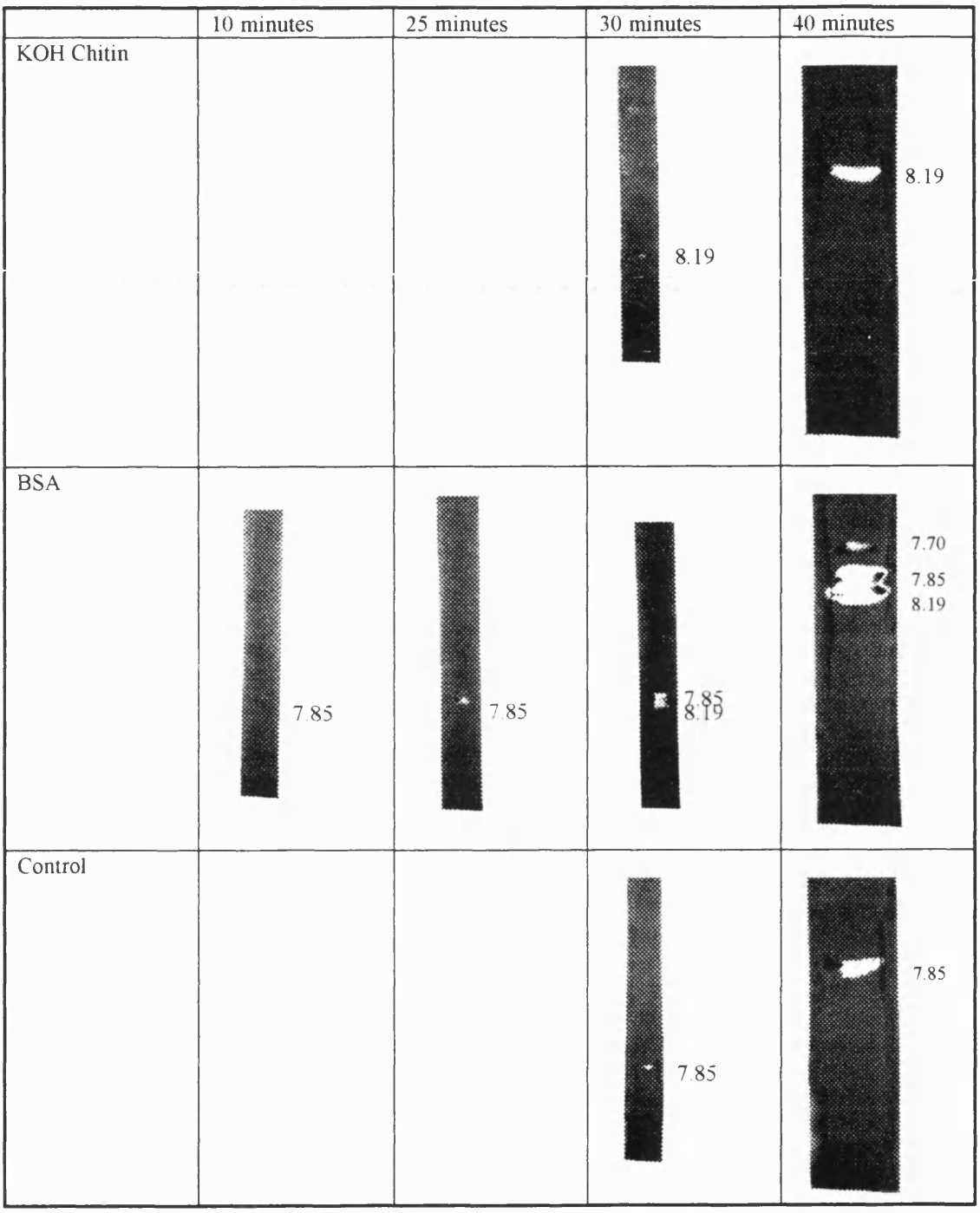


Figure 3.30. Degradation of overlay with time. Focused lanes of the gel were overlaid with moist x-ray film and degradation zones were noted and photographed as they developed. Key time points are shown for locust cuticle, practical grade chitin (from crab shells), deproteinised chitin (KOH treated) and control - basal salts alone. Similar results were found when the experiment was repeated with three replicate samples.

In BSA cultures, a similar pattern to that of cuticle cultures was observed, in that three forms, pI 7.7, 7.85 and 8.19 were produced initially. Isoform pI 8.95 was much slower to degrade the overlay. In control cultures degradation was only observed at pI 7.85. . No degradation of the overlay was observed at pI 9.47

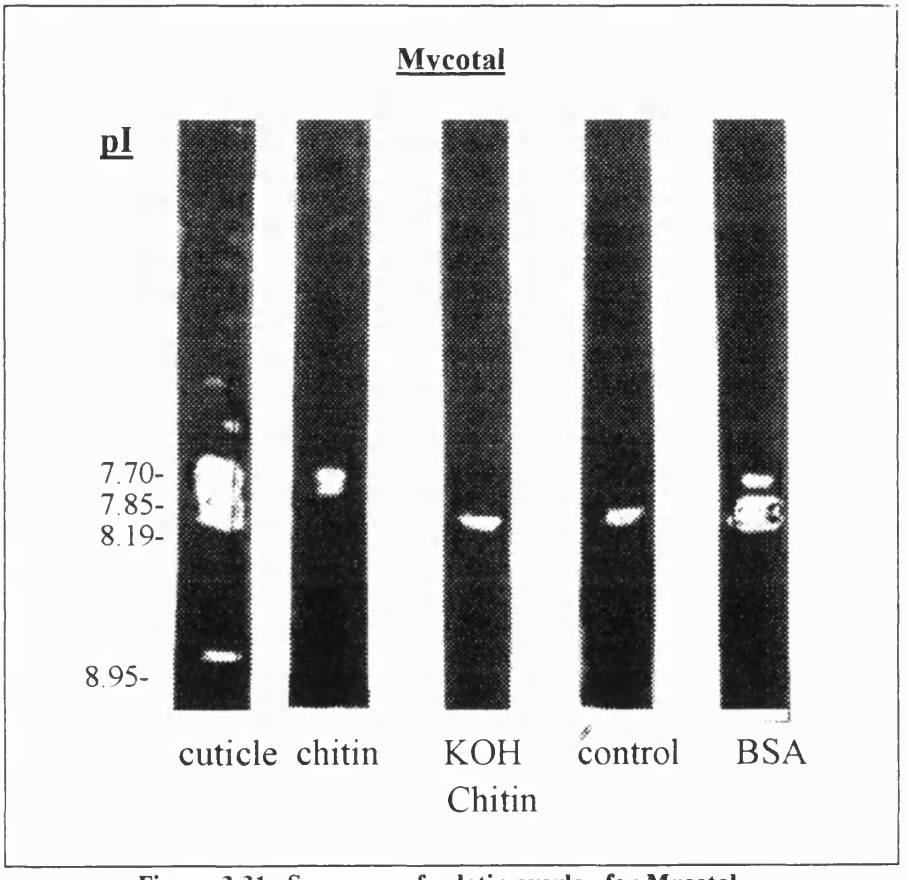
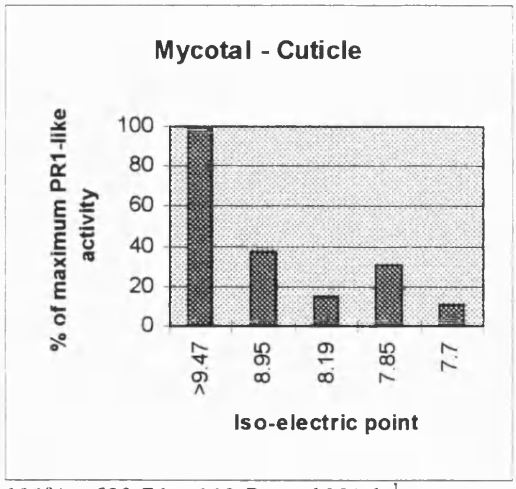


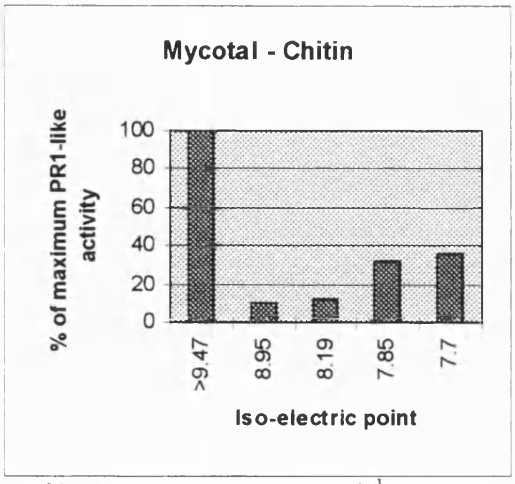
Figure 3.31. Summary of gelatin overlay for Mycotal

d. PR1-like activity - Mycotal

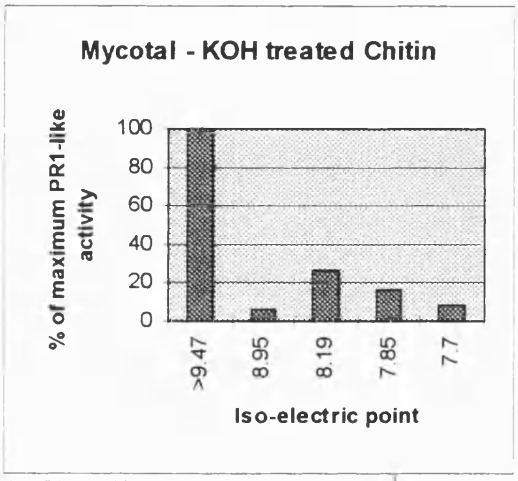
For each substrate tested, as found for Vertalec, Mycotal produced the highest chymotrypsin activity in the most basic gel slice, pI 9.47, this protease, however, was not seen to degrade the gelatin overlay. On insect cuticle, 4 more proteases were produced which showed activity against the chymotrypsin substrate, pI 8.95, 7.85, 8.19 and lastly 7.7 (in descending enzyme activity). Interestingly the protease at pI 8.95 showed considerable activity against the PR1-like substrate, yet it was slow to degrade the gelatin overlay. In chitin containing cultures, the PR1-like enzyme with pI 8.95 was repressed and that of pI 7.7 appeared to be enhanced. Conversely next to that of the most basic form, the PR1-like activity of isoform pI 8.19 was enhanced in cultures containing deproteinised chitin. In BSA containing cultures, all forms of the protease observed in cuticle cultures were present. Finally, when transferred into basal salts alone, all forms of PR1-like enzyme appeared to be repressed with the exception of that at pI $\geq$9.47.



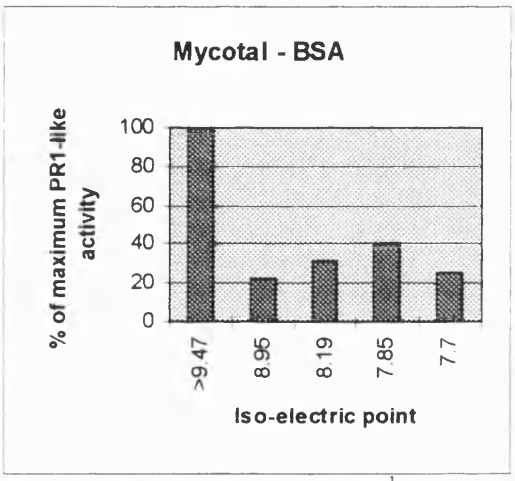
100% = 523.76 ± 102.7 nmol NA h^{-1}



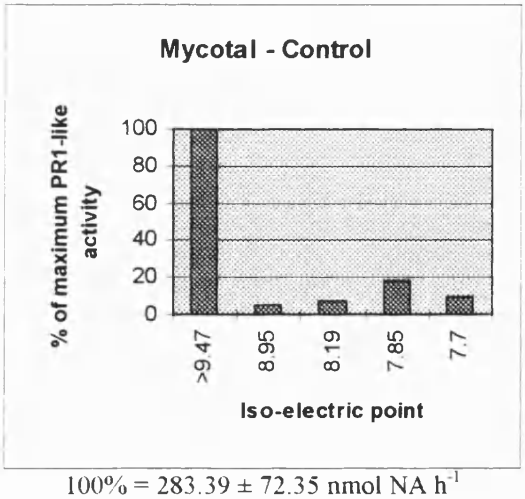
100% = 458.36 ± 95.9 nmol NA h^{-1}



100% = 463.08 ± 92.58 nmol NA h^{-1}



100% = 414.53 ± 101.7 nmol NA h^{-1}



100% = 283.39 ± 72.35 nmol NA h^{-1}

Figure 3.32. Isoforms of a PR1-like enzyme produced by Mycotal on different induction conditions. Activity of the excised gel slices against the chymotrypsin substrate is expressed as a percentage of the highest activity (100%) recorded for each sample lane. The actual release of nitroalanine per hour and standard deviation are shown for 100% as a means for comparison (n=3).

Summary

Vertalec

Estimated pI				
Cuticle	Chitin	KOH treated chitin	Bovine Serum Albumin	Control (basal salts only)
≥ 9.47	≥ 9.47	≥ 9.47	≥ 9.47	≥ 9.47
9.10				
8.83	8.83			
8.62	8.62	8.62		8.62

Mycotal

Estimated pI				
Cuticle	Chitin	KOH treated chitin	Bovine Serum Albumin	Control (basal salts only)
≥ 9.47	≥ 9.47	≥ 9.47	≥ 9.47	≥ 9.47
8.95			8.95	
8.19	(8.19 - low levels only)	8.19	8.19	
7.85	7.85	(7.85 - low levels only)	7.85	(7.85 low levels only)
7.7	7.7		7.7	

3.1.5.4 Iso-electric separation of PR1-like enzymes produced by Vertalec when an established biomass of fungus is transferred basal salts supplied with N-acetyl glucosamine in a restricted manner

Aliquots of concentrated filtrate containing 50 µg protein from a Vertalec culture supplied with N-acetyl glucosamine at a rate of 20-25 µg GlcNAg ml^{-1} h^{-1} for 16 hours, were loaded onto a broad range iso-electric focusing gel. Gelatin overlays and gel slice assays were performed as previously.

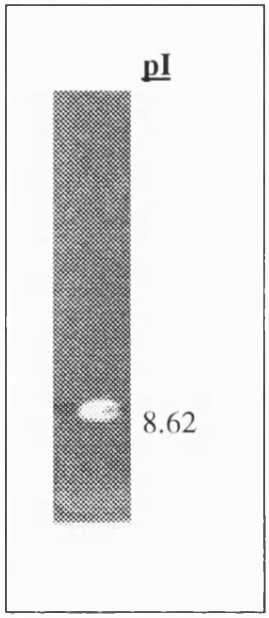
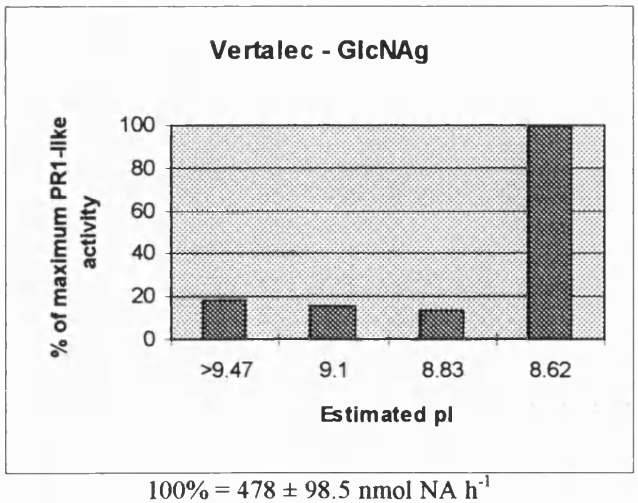


Figure 3.33 Degradation of gelatin overlay by proteolytic activity from N-acetyl glucosamine Vertalec cultures. The position of the degradation zone was noted and the pI of the protease estimated by comparison to broad range pI markers run on the same gel and stained with Coomassie blue. No further degradation bands were observed.



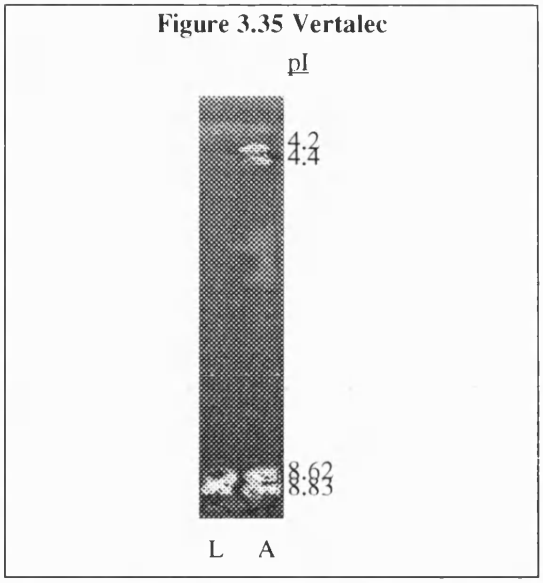
100% = 478 ± 98.5 nmol NA h^{-1}

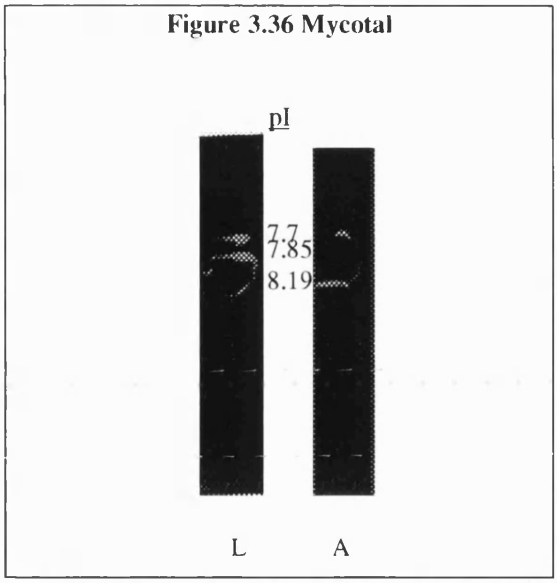
Figure 3.34. Isoforms of a PR1-like enzyme produced by Vertalec on N-acetyl glucosamine after 16 hours. Activity of the excised gel slices against the chymotrypsin substrate is expressed as a percentage of the highest activity (100% recorded for the sample lane. The actual release of nitroalanine per hour and standard deviation are shown for 100% as a means for comparison.

In cultures of Vertalec where N-acetylglucosamine was supplied in a restricted manner as the sole source of carbon and nitrogen, PR1-like activity was attributable to one isoform at pI 8.62.

3.1.5.5 A comparison of isoforms of protease produced by Vertalec and Mycotal on locust and aphid cuticle

Aliquots of concentrated supernatants containing 50 µg protein from 24h aphid or locust cuticle cultures were loaded onto a broad range iso-electric focusing gel. Gelatin overlays and gel slice assays were performed as previously.





Figures 3.35 and 3.36 Degradation of gelatin overlay by proteolytic activity from cultures of *V.lecanii* on locust (L) or aphid cuticle (A). The position of the degradation zone was noted and the pI of the protease estimated by comparison to broad range pI markers run on the same gel and stained with Coomassie blue. (Photographs for each isolate are not to comparative scale).

The pattern of degradation of the gelatin overlay and gel slice assays for PR1-like activity (not shown) were very similar within aphid and locust cuticle cultures for both Vertalec and Mycotal, although the proteases from Mycotal on aphid cuticle were slower to degrade the gelatin overlay than those on locust cuticle. Furthermore, the relative activities of PR1-like enzymes were found to be similar to that observed in section 3.1.5.1. However, two acidic proteases were observed in aphid cuticle cultures of Vertalec, that were not observed in locust cuticle cultures, at pI 4.2 and 4.4. These proteases did not have activity against the PR1 or PR2 substrates.

3.1.5.6 The ability of PR1-like enzymes from 3 lead strains of *V.lecanii* to degrade aphid and locust cuticle

Given that the same isoforms of PR1 enzyme had been observed in aphid and locust cuticle cultures of Vertalec and Mycotal, and filtrate from locust cuticle cultures was more readily available, this was used in digestion comparison experiments.

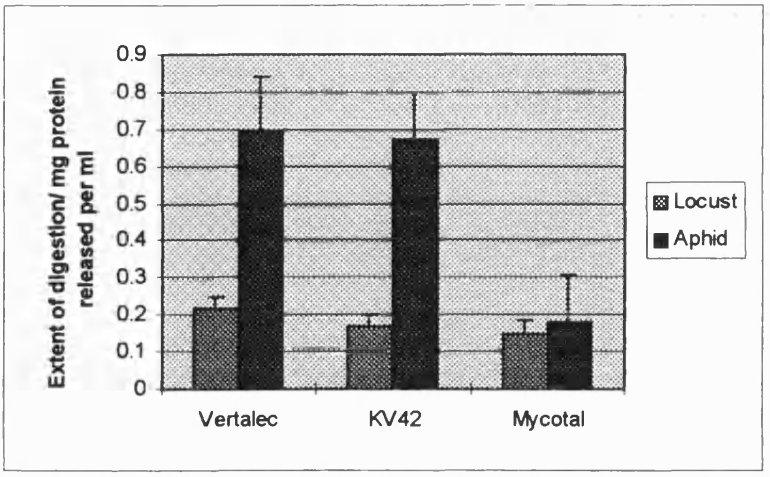
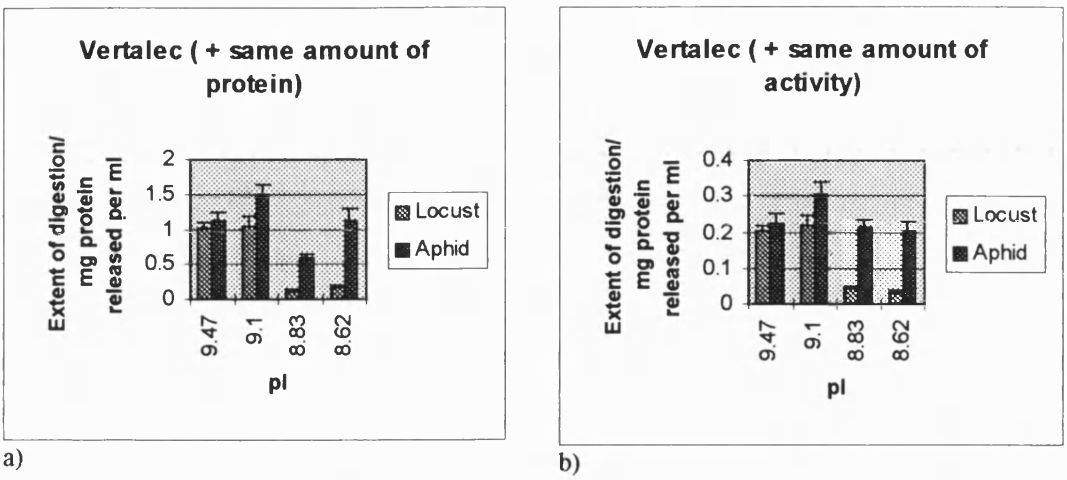


Figure 3.37. A comparison of the amount of protein released from locust and aphid cuticle after an overnight incubation with crude filtrate from three strains of *V.lecanii*. 50µg protein from filtrates of locust cuticle cultures of Vertalec, KV42 and Mycotal was incubated with 5mg cuticle in buffer overnight at 28°C. The amount of protein released was estimated by UV detection and is shown as the mean of 3 replicate incubations (standard deviations are shown).

A similar amount of protein was hydrolysed from locust cuticle after overnight incubation with filtrates from three isolates of fungus. However, enzymes from Vertalec and KV42 cultures, digested 3-4 fold more protein from aphid cuticle than from on locust cuticle. This was not the case with Mycotal cultures.

A comparison was then made between the digestive capability of different isoforms of PR1-like enzyme for each of the three isolates. Isoforms of PR1-like enzyme were semi-purified by eluting the protein from the IEF gel at the appropriate pI. Experiments were carried out in two ways. Cuticle digestion was determined when the same amount of enzyme protein (20µg) was added to the reaction mixture, and when

the same amount of PR1-like activity was added to the cuticle (that which releases 10nkat NA min^{-1} ml^{-1} from N-Suc-Ala-Ala-Pro-Phe-pNA).



a) b)

Figure 3.38. A comparison of the amount of protein released from locust and aphid cuticle after an overnight incubation with isoforms of PR1-like enzyme as semi-purified from Vertalec cuticle cultures. 20μg protein at the iso-electric points shown (a) and equal amounts of PR1-like activity (that which causes the release of 10nkats NA per min per ml) of each isoform (b) was incubated with 5mg cuticle in buffer overnight at 28°C. The amount of protein released was estimated by UV detection and is shown as the mean of 3 replicate incubations (standard deviations are shown).

In the case of Vertalec, the most basic PR1-like isoform ($\geq$ 9.47) and that of pI 9.1 appeared to be the most efficient at digesting, and releasing protein from locust cuticle, with isoforms 8.83 and 8.62 being relatively poor (Figure 3.38). These differences were not due to differences in PR1-like activity as a similar pattern was observed in incubation conditions supplemented with the same amount of enzyme activity.

On aphid cuticle a similar amount of protein was released to that on locust cuticle by pI $\geq$9.47. Isoform pI 9.1 seemed to be slightly more efficient at digesting the aphid cuticle than the locust cuticle. The release of protein from aphid cuticle by isoforms 8.83 and 8.62 was far higher than on locust cuticle, and was comparable to the efficiency of digestion by the other isoforms when the same amount of enzyme activity

was supplied. When the same amount of protein of each isoform was added to the incubation conditions, the isoform of pI 8.83 was the weakest digester of aphid cuticle, suggesting that this is a reflection of lower specific activity rather than the cuticle degrading efficiency of the enzyme.

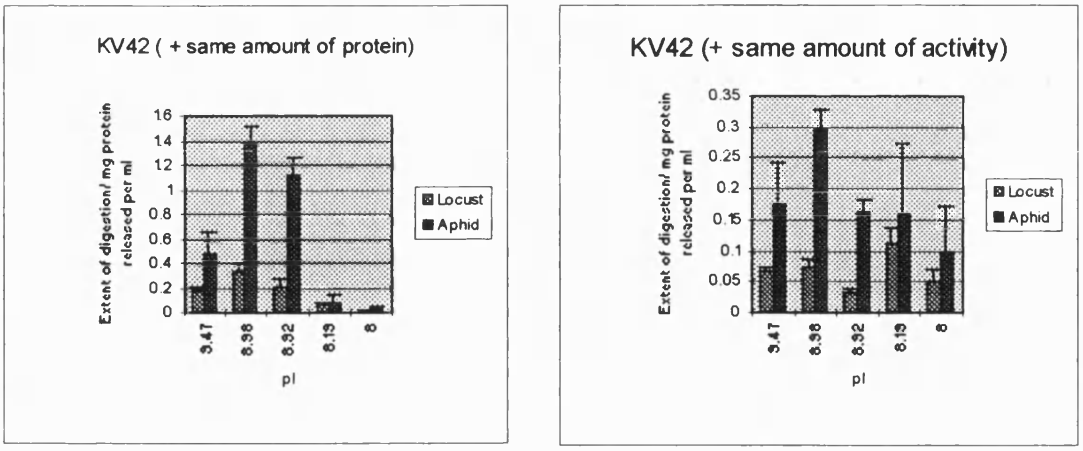


Figure 3.39. A comparison of the amount of protein released from locust and aphid cuticle after an overnight incubation with isoforms of PR1-like enzyme as semi-purified from KV42 cuticle cultures. 20µg protein at the iso-electric points shown (a) and equal amounts of PR1-like activity (that which causes the release of 10nkats NA per min per ml) of each isoform (b) was incubated with 5mg cuticle in buffer overnight at 28°C. The amount of protein released was estimated by UV detection and is shown as the mean of 3 replicate incubations (standard deviations are shown).

When the same amount of protein of each isoform of KV42 was included in the incubation conditions, digestion of locust cuticle was highest by isoforms ≥9.47, 8.98 and 8.92, but was negligible for isoforms pI 8.19 and 8.0. When similar activities were included all efficiencies of digestion appeared to be similar, with 8.19 releasing most protein.

The amount of protein released from aphid cuticle by isoforms pI ≥9.47, 8.98 and 8.92 was much higher than that released from locust cuticle. Isoforms pI 8.19 and 8.0 were equally efficient against the two cuticles. A similar pattern of enzyme activity was found when experiments were conducted with equivalent enzyme activities.

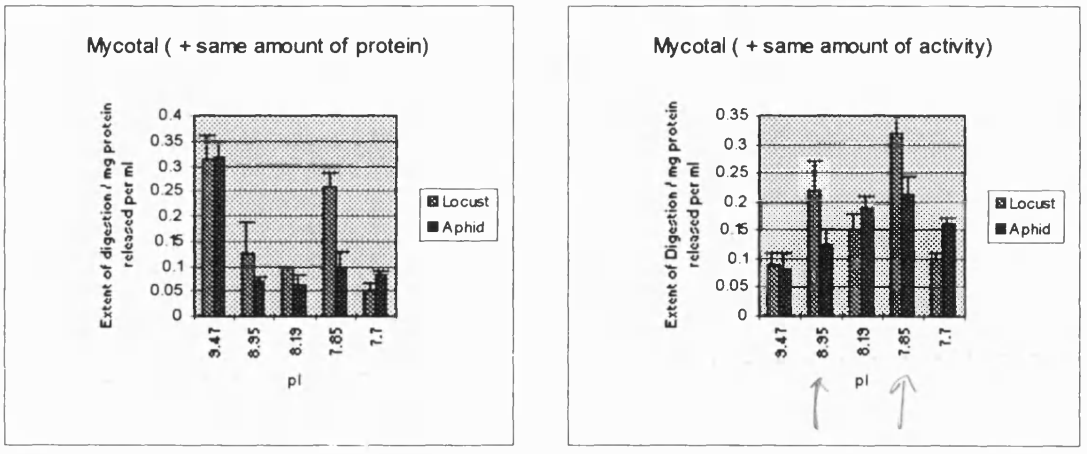


Figure 3.40. A comparison of the amount of protein released from locust and aphid cuticle after an overnight incubation with isoforms of PR1-like enzyme as semi-purified from Mycotal cuticle cultures. 20μg protein at the iso-electric points shown (a) and equal amounts of PR1-like activity (that which causes the release of 10nkats NA per min per ml) of each isoform (b) was incubated with 5mg cuticle in buffer overnight at 28°C. The amount of protein released was estimated by UV detection and is shown as the mean of 3 replicate incubations (standard deviations are shown).

All isoforms of PR1-like enzyme produced by Mycotal were able to digest locust cuticle to release protein. When the same amount of protein was supplied, the most efficient isoforms were pI 9.5 and 7.85, however this appeared to be due to their high specific activity. When same enzyme activities were added to the cuticle, pI 8.95 and 7.85 were the largest releasers of protein. Digestion of aphid cuticle by isoforms pI $\geq$9.47 and pI 8.19 was very similar to that of locust cuticle. Isoforms 8.95 and 7.85 appeared to release more protein from locust cuticle than on aphid cuticle, and for pI 7.7 this situation appeared to be reversed.

3.2 DISCUSSION

Given the suggested importance of extracellular proteinases in the disease process of entomopathogenic fungi, elucidating the mechanisms regulating their secretion is central to understanding pathogen growth and development in the host (St Leger et al. 1997 & 1998). The objectives of these *in vitro* studies were to investigate the production of proteases by five different isolates of *Verticillium lecanii*. Isolates of a fungus grown under the same conditions, although morphologically similar, may express different physiological and developmental responses (St Leger et al. 1992a,c) thus, here attempts were made to identify possible contrasting strategies of proteinase regulation between given strains of *V.lecanii*.

All 5 of the isolates of *V.lecanii* produced enzymes with activities against the chymoelastase (PR1) and trypsin (PR2) substrates, N-Suc-$(Ala)_2$-Pro-Phe-pNA and Bz-Phe-Val-Arg-pNA, respectively (St Leger et al. 1987c) when grown on locust cuticle as the sole source of carbon and nitrogen over an eight day period. Levels of production of PR1 and PR2-like enzymes differed between isolates, being highest in Mycotal and KV42 cultures respectively. High absolute levels of activity may not be the only attribute of cuticle degrading proteinases that promotes host penetration. The timescale of production may also be important. Isolate KV54 displayed a peak of PR1-like activity on day 4 compared to day 7 for Mycotal - this early synthesis of PR1 may allow accelerated host invasion and faster kill.

Trypsin (PR2-like) production peaks 1 day after the subtilisin (PR1-like) enzyme in Vertalec, KV42 and KV54 cultures and simultaneously in KV22 and Mycotal. This

pattern of production is the converse of that in cultures of isolates of *Metarhizium anisopliae* where PR2 consistently precedes PR1 (Kershaw, 1993; Gillespie et al. 1998). The later production of PR2 by *V.lecanii* suggests a role for this enzyme in the hydrolysis of the products of PR1 cuticle degradation, rather than in providing inducers of PR1 synthesis, as suggested for *M.anisopliae* (Paterson et al. 1994b.)

The ability to produce cuticle-degrading proteolytic enzymes *per se* does not distinguish entomopathogens from other fungi, however the ability to regulate production in response to host specific cues, such as from the insect cuticle, could be important in adaptation to a pathogenic life-style (St Leger 1995). The function of the enzyme appears to dictate its regulatory strategy. In *M.anisopliae* and *B.bassiana*, chitinase, required for only a short period during penetration of the cuticle is tightly regulated by chitin degradation products (St Leger et al 1986c, Smith and Grula 1983) however since proteases are important for providing nutrients before and after penetration, they are produced constitutively but subject to multiple regulatory circuits. For *M.anisopliae* production of PR1 is controlled by nutrient limitation (St Leger et al. 1988c, 1992c), developmental regulation (St Leger et al. 1989b) and cuticle induction (Paterson et al 1994a). A number of lines of evidence suggest that the PR1-like enzymes of *V.lecanii*, isolate Vertalec, are also induced by insect cuticle. Firstly, chymoelastase activity on insect cuticle was considerably higher (up to 20fold more) than in basal salts alone; secondly, this difference was not accountable to a change in biomass and finally, dialysis of the medium indicated that different levels of activity were due to enzyme production and not environments that inhibited or enhanced the enzyme's activity.

The PR1-like enzymes of Vertalec appear to be under the control of both the chitin and, to a lesser extent, protein, components of the insect cuticle -high levels of activity were observed on chitin, de-proteinised chitin and BSA as well as cuticle. In contrast deproteinised cuticle and chitin alone had no effect on induction of PR1 of *M.anisopliae* (ME1) and Bovine Serum Albumin, completely repressed PR1 production (Paterson et al. 1994a & b). Paterson and Co-workers (1994b) concluded that this enzyme was specifically induced by a proteinaceous component of the cuticle - particularly the alanine rich peptides found within host insect cuticle. .Elastin, like cuticle, is an insoluble substrate with a high proportion of alanine residues (Hojrup et al. 1986) and this substrate did promote some PR1 production greater than the controls in Vertalec cultures, and for *M.anisopliae* ME1 (Paterson et al. 1994a), however not to the extent of the insect cuticle, suggesting specific induction by actual cuticular components.

Although chitin had an inductive effect on Vertalec, other non-protein substrates such as cellulose and urea did not promote PR1-like activity, again suggesting some level of specific induction by the insect cuticle. The PR2-like enzymes of Vertalec were similarly induced by cuticle, although activity did not reach anywhere near the levels found for PR1. PR2-like enzymes did not appear to be under such specific control as PR1; they were induced by a number of proteinaceous substrates such as BSA, gelatin and elastin, as found for *M.anisopliae* ME1 (Paterson et al. 1993).

Most studies of enzyme regulation of *M.anisopliae* have been conducted on a single isolate ME1 (St Leger et al 1986c, 1988c, Paterson et al. 1994a,b), this does not

consider isolate variability. All isolates of *V.lecanii* tested in this study, like *M.anisopliae* ME1 (Paterson 1994a) produced PR1-like enzymes that were induced by insect cuticle as the sole source of carbon and nitrogen. However, these 5 isolates did not exhibit such specific regulation as *M.anisopliae* (ME1) and significant differences were found between them with respect to PR1. Since only relatively low levels of PR2-like enzyme were produced by *V.lecanii*, Vertalec, isolate comparison experiments were not conducted.

PR1-like activity in Mycotal cultures was produced substantially above control on chitin and deproteinised chitin, however, considerably more activity was produced on cuticle and Bovine Serum Albumin. Thus in contrast to Vertalec and more in common with the trypsin PR2 from *M.anisopliae* ME1 (Paterson et al. 1993) the over-riding regulation for Mycotal PR1 is induction by non-specific protein. The high levels of production of proteinase by Mycotal emphasise its possible importance in breaking down the protein of the procuticle, exposing the chitin fibrils to other enzymes.

Due to the availability of locust cuticle, this was used in the transfer experiments described. However, given that these isolates of *V.lecanii* showed differing levels of regulation in response to chitin, and the protein:chitin ratios differ with each insect cuticle (Hackman 1974, Neville 1975), it was important to compare induction by aphid as well as locust cuticle.

St Leger et al. (1997) suggest that the array of enzymes produced by insect and plant pathogens and their mode of regulation is dependent on the composition of the

integument of their hosts.. However, knowledge on enzymic adaptation to different insect cuticles and the mechanisms for host specificity is still limited.

For Vertalec and Mycotal, aphid and whitefly isolates respectively, both aphid and locust cuticle induced the production of chymoelastase. For Vertalec, there was no significant difference between activity on the two cuticles. Since this isolate is principally induced by chitin, presumably these PR1-like enzymes are induced non-specifically by chitin components common to insects as diverse as aphids and locusts. For Mycotal, an isolate principally induced by protein, at 16 h, comparable activities were found on the two cuticles, however at 24h, activity was significantly higher on locust than on aphid. Future experiments should utilise cuticle from whitefly, the original host of Mycotal.

Isolates of *M.anisopliae* from Lepidopteran and Coleopteran hosts did not appear to exhibit lower levels of PR1 production on non-host cuticles (Gupta et al. 1991). In contrast, Gupta et al. (1992) working on *B.bassiana* and El-Sayed et al. (1993) on *Nomuraea rileyi* reported that PR1 *was* induced differently by different cuticles.

Paterson et al. (1994b) suggest that induction of PR1 from *M.anisopliae* only occurs during infection of hosts whose cuticular protein(s) contain the inducing peptide sequence. In this study both locust and aphid cuticle promote PR1-like production by *V.lecanii* isolates, suggesting a common inducer in these two very different insects.

Cuticle-degrading enzymes could contribute to host specificity of entomopathogens, either through cuticle-specific activity or cuticle-specific production (see later).

All the aphid isolates of *V.lecanii* tested, *viz* Vertalec, KV22, KV42 and KV54, produced PR1-like enzymes that were induced by chitin. Vertalec, although not displaying high levels of activity in cultures containing impure practical grade chitin, produced highly significant amounts of PR1 once chitin was stripped of trace protein using KOH. KV22 and KV42 were less sensitive to the presence of trace protein in chitin cultures. BSA also supported substantial activity in cultures of Vertalec and KV22, although not to the extent of the whitefly active isolate Mycotal. In common with *M.anisopliae* ME1 (Paterson et al.1994a), BSA was repressive for KV54 and KV42.

At first sight, it seems counter intuitive that proteases should be induced by chitin. However, given that the cuticle consists primarily of chitin fibrils embedded in a protein matrix (Neville, 1984) and that fungal chitinases are also usually induced by chitin (St Leger et al. 1986c, Smith and Grula 1983) co-ordinated regulation of enzymes that hydrolyse the two main constituents of cuticle may prove most efficient under some circumstances. This is not the first time that fungal proteases have been shown to be induced by chitin or components of chitin. PRB1 of *Trichoderma harzianum* is induced by chitin in fungal cell wall preparations and is repressed in the presence of casein or BSA (Geremia et al. 1993).

Chitin *per se* cannot be the inducer because it is an insoluble polymer. Geremia et al. (1993) speculated that one signal for PRB1 production could be a chitin degradation product. N-acetylglucosamine (GlcNAg), the principal monomeric constituent of chitin, has been shown to be the major product of chitin hydrolysis by *M.anisopliae* (St Leger et al.1986c). Synthesis of extracellular chitinase in *M.anisopliae* and *B.bassiana*

is regulated by levels of GlcNAg through an inducer-repressor mechanism (St Leger et al. 1986c, Smith & Grula 1983). One isolate of *Beauveria bassiana* produced PR1-like enzymes when supplied with low levels of N-acetyl glucosamine (Bidochka and Khachatourians 1988a). The authors proposed a simple regulatory system as follows: during cuticle penetration basal levels of extracellular proteases hydrolyse proteins, exposing chitin fibrils, these are degraded to GlcNAg by basal levels of chitinase. Low levels of GlcNAg induce chitinase and protease whereas high levels are repressive. The application of this hypothesis to *V.lecanii* proteases was tested using diffusion capsules (Pirt 1971). These allow restricted presentation of GlcNAg to the fungus and thus prevent catabolite repression. A comparison was made between Vertalec, in which serine protease production is induced by chitin, and Mycotal, in which serine protease production is predominantly induced by protein. Mycotal did not produce PR1-like activity when GlcNAg was supplied to the medium in a restricted or non-restricted manner. Levels on cuticle remained high. For Vertalec, high concentrations of GlcNAg repressed PR1 levels to those on basal salts only, however, with a restricted supply of GlcNAg, PR1-like activity was comparable to that found on cuticle.

PR1-like activity is not needed under GlcNAg rich conditions, when the fungus has adequate nutrients. These results are consistent with the application of the hypothesis of Bidochka and Khachatourians (1988a), that protease is only produced under low levels of GlcNAg, to the PR1-like protease of Vertalec. Mycotal PR1, like that of *M.anisopliae*, is induced independently of chitin degradation.

Chymoelastase production by *V.lecanii* is subject to multiple regulatory circuits of which induction is just one. Three of the isolates tested, Vertalec, KV42 and KV22 produced PR1-like enzymes in basal salts alone. St Leger et al. (1988c, 1991c) also observed a substantial basal level of PR1 synthesis by *M.anisopliae* under conditions of carbon and nitrogen starvation. All *V.lecanii* isolates also displayed some measure of catabolite repression when grown on locust cuticle supplemented with low molecular weight carbon and/or nitrogen. PR1 from *M.anisopliae* was repressed by a soluble source of carbon, but predominantly by low molecular weight nitrogen (Paterson et al. 1994a). By contrast, PR1-like enzymes of Vertalec, KV54 and KV22, showed a level of repression by nitrogen but were predominantly repressed by carbon. A comparable situation occurs for the control of the extracellular protease of the mosquito-parasitising fungus *Lagenidium giganteum* (Dean and Domnas 1983). Mycotal appears to exhibit control of serine protease production equally by carbon and nitrogen repression. Dual control also occurs with KV22, though in this case nitrogen repression is short lived.

An insect's susceptibility or resistance to an individual isolate will be determined partly by the nutrient conditions on the surface of the cuticle prior to infection and the isolate's sensitivity to catabolite repression (St Leger et al. 1989b). KV42 was the only isolate of *V.lecanii* tested in which PR1-like enzymes were not subject to repression by a carbon *or* nitrogen. This is exciting, given that in aphid hosts the endogenous nutrients of the cuticle may be augmented by sugars from excreta (honey dew). Absence of carbon catabolite repression may accelerate enzyme production and promote early host penetration and prevent the extensive growth on the surface observed for some isolates of *V.lecanii* (Schreiter et al. 1994). Isolates more sensitive

to catabolite repression, may be less virulent under nutrient rich conditions, for example, addition of nutrients to the cuticle surface of *Manduca sexta* prevented infection by *M.anisopliae* ME1 (St Leger et al. 1989b).

Appressorium formation and PR1 expression in *M.anisopliae* are repressed by alanine and glucose, suggesting a co-ordinate regulation by catabolite repression (Clarkson and Charnley 1996). *V.lecanii* does not produce an obvious appressorium. However, a differentiation process must take place prior to host penetration which leads to the switching on of pathogenicity genes. Some isolates of *M.anisopliae* isolated from homopteran pests, produce appressoria which are not glucose repressed (St Leger et al 1992b). Perhaps the comparable biochemical differentiation process in KV42 is also not repressed by soluble carbon.

All isolates of the fungus exhibited almost total repression of PR1-like activity when *both* additional nitrogen and carbon were present in cuticle cultures. This suggests, that catabolite repression for *V.lecanii* could conserve serine protease production in environments where it is not required, or where the release of degradation products from the cuticle exceeded fungal requirements (St Leger 1995). In fact, the majority of cuticle degrading enzymes produced by *M.anisopliae* were repressed by either carbon or nitrogen, with the exception of N-acetylglucosaminidase (reviewed by St Leger 1995).

Catabolite de/repression mechanisms also occur in the absence of insect cuticle - the presence of soluble carbon repressing any PR1-like enzymes produced by Vertalec and KV22 in basal salts alone. However, in control cultures of KV42 at 36 h, low levels of

activity were found when additional carbon *and* nitrogen were supplied. It is possible that at 36 h the fungus has metabolised the carbon and nitrogen supplement and that PR1-like activity is beginning to be induced by a starvation response. When additional soluble carbon is supplied to KV42 cultures on bovine serum albumin, PR1-like activity actually seems to be enhanced, and cultures of Vertalec at 16 h seem to be less sensitive to soluble carbon. These results have implications for the expression of proteases in aphid haemolymph (see chapter 4).

To date, studies on the regulation of proteases by entomopathogenic fungi have failed to take into account isozyme variation. *Metarhizium anisopliae*, isolate ME1, produces four basic isoforms of PR1 of pIs 10.2, 9.8, 9.3 and 9.0 (St Leger et al. 1994b), two of which have been characterised as PR1a and PR1b ((St Leger et al. 1992c and Joshi et al. 1997 respectively).). In the present work, isoforms of chymoelastase were identified from three isolates of Vertalec, Mycotal and KV42. Previous studies on isolates of *V.lecanii* from the codling moth and the aphid, *M.sanborni* indicated very similar profiles of PR1-like enzymes when analysed by preparative iso-electric focusing, with two main peaks at pI ~10 and pI~7 (St Leger et al 1987b). Here, clear inter-isolate differences were observed not only in the isoforms produced but in their regulation.

The predominant isoform produced by each of these three fungal isolates was a very basic form, pI $\geq$9.47 at the limit of the resolution of the gel. High chymoelastase activity was recovered from this section of the gel for all three isolates, however, for KV42 and Mycotal, this protease was unable to degrade gelatin - indicating a difference to the isoform produced by Vertalec. Some PR2-like activity was also

detected at pI ≥9.47, but this isoform appeared to be a single band on an IEF gel and SDS-PAGE gel (chapter 4). Furthermore, inhibitor studies using each substrate revealed a similar pattern of inhibition, suggesting the presence of a single enzyme with overlapping activities rather than two enzymes. Samuels et al. (1990) similarly found proteases produced by a number of *Erynia* spp. that had mixed chymotrypsin/trypsin activity. Inhibitor studies indicated that this form from all isolates of *V.lecanii* tested was predominantly a chymotrypsin, although for Vertalec, it appeared to have some properties of a conventional trypsin.

For Mycotal and Vertalec, this very basic isoform was not only produced on aphid and locust cuticle, but also on chitin, KOH treated chitin, BSA and even in the basal salts control. For Vertalec, this isoform was repressed when N-acetyl glucosamine was supplied as sole nutrient in a restricted manner and, as were all other isoforms, on addition of soluble carbon to cuticle cultures. The equivalent form of the enzyme for KV42 as produced on cuticle, was not repressed by carbon, only carbon *and* nitrogen together. Thus, although these two isolates have a PR1-like enzyme with similar pI, the isoforms appear to be regulated differently.

With the exception of this basic form of the enzyme, each isolate studied, produced a very different profile of PR1-like enzymes on insect cuticle as the sole source of carbon and nitrogen. Vertalec produced 3 basic proteases with PR1-like activity at pI 9.1, 8.83 and 8.62. Inhibitor studies again suggested that they were predominantly chymotrypsins and they responded to the chymotrypsin/trypsin inhibitor turkey egg white, although isoform pI 9.1 was less sensitive. All forms were produced on locust and aphid cuticle, indicating that specificity does not operate at the level of production.

However, there was some indication of differential regulation between isoforms. On BSA, the low levels of PR1-like activity were attributable to the most basic form (≥9.47), all others forms were repressed.

Isoform of pI 9.10 was only produced on insect cuticle. It was not derepressed by starvation nor was it specifically induced by chitin or protein. It appears to be induced specifically by a component(s) of the insect cuticle itself. Furthermore, this form shows sensitivity to both carbon and nitrogen catabolite repression. It was completely repressed in the presence of carbon, but unlike the other forms, was sensitive to nitrogen. The repression of this isoform accounts for the loss of activity in cuticle cultures supplemented with nitrogen. The remaining two enzymes were not repressed by the addition of nitrogen, but like pI ≥9.47, were completely repressed by carbon. pI 8.83 was only produced in cuticle cultures or those containing practical grade chitin, but not de-proteinised chitin. It is not controlled by a starvation response alone, but rather is induced by some proteinaceous component(s) of the insect cuticle. The least basic of all proteases, pI 8.62 was produced in all test cultures with the exception of BSA. As one of the main isoforms produced on deproteinised chitin, it was the only form produced on N-acetyl glucosamine. Thus isoform pI 8.62 is regulated independently from all other forms of enzyme, by a possible breakdown product of chitin.

Very few authors investigating regulation of extracellular enzymes produced by entomopathogenic fungi have taken into consideration the existence of multiple forms of the target enzyme, and thus the possibility of different modes of regulation for each isoform. The most comprehensive work on regulation of proteases produced by

M.anisopliae and *B.bassiana*, to date, by authors such as Paterson et al. (1994a,b), St Leger et al. (1988c, 1991c) and Bidochka and Khachatourians (1988a,b) have assumed co-ordinate regulation of all PR1-like enzymes produced. This is obviously not the case for these isolates of *V.lecanii*.

In support of the findings for *V.lecanii*, the most complex number of PR1 and PR2 isoforms produced by *M.anisopliae* was found on insect cuticle as the sole source of nitrogen. No further forms were observed on any other substrate tested (St Leger et al. 1994b). However, the number of PR1 isoforms on cuticle diminished with time. Furthermore only 2 forms were produced, albeit at diminished levels, on elastin or cellulose. St Leger et al. (1994b) attributed this to differing sensitivities to carbon and nitrogen derepression, rather than differential induction. Thus for *M.anisopliae* isoforms may be differentially expressed or have different stabilities dependent on the culture media. The authors suggested that a possible reason for the existence of PR1 as isoforms may lie in the need for genetic regulation to provide developmental or catabolite specific expression during different stages of host invasion. To make any real conclusions, however, a full investigation into the induction and catabolite de/repression mechanisms of PR1 isoforms is needed.

Research into this area is also very limited for plant pathogenic fungi. Those studies where authors have attempted to identify different forms, suggest that isoforms of extracellular enzymes involved in pathogenesis or saprotrophy are regulated by the same mechanisms. The broad bean rust fungus, *Uromyces viciae-fabae*, for example, produced four isoenzymes of pectin methylesterase which were all similarly regulated and differentiation specific (Deising et al. 1995) and 2 isoforms of cutinase produced

by *Alternaria brassicola* were both induced similarly by cutin monomers (Fan et al. 1998). Some workers have, however, observed differential regulation of members of enzyme gene families. The cutinolytic esterases of *A.brassicola*, in contrast to the cutinases described previously, were induced by cutin freed from wax and not cutin monomers (Fan et al. 1998). The authors suggest that these different strategies of regulation imply the existence of a regulatory switch - that the cutinolytic esterases are involved in pathogenesis, and the cutinases are important for the saprophytic utilisation of cutin. The pectate lyases of the phytopathogenic fungus, *Fusarium solani* are regulated differently. Two genes have been characterised, one is induced by pectin and repressed by glucose the other is produced constitutively and is not subject to glucose repression (Guo et al. 1995). The authors suggest that differential regulation of these 2 pectate lyase enzymes could be important during host penetration - for example, the constitutive production of one enzyme could generate cell wall degradation products which may induce the expression of other pectin degrading enzymes. The absence of such breakdown products may prevent the induction of other pectate lyase enzymes, thus conserving them in environments where they are not needed.

It remains to be seen whether or not the PR1-like enzymes produced by these isolates of *V.lecanii* are products of the same gene or different genes and work is needed to fully characterise each enzyme to try to understand why they are regulated differently. However, it would appear that the identification of different inductive strategies of different forms of PR1-like enzyme is largely novel within insect and plant pathogenic fungi.

The whitefly isolate, Mycotal, produced 4 isoforms of enzyme with PR1-like activity on insect cuticle in addition to that at pI ≥9.47, *viz* pI 8.95, 8.19, 7.85 and 7.7. The activities at pI 8.19 and 7.7 were very low in comparison to the other forms. None of the isoforms of Mycotal showed any activity towards the PR2-like substrate. All forms were repressed by chymostatin, indicating chymotrypsin like properties, However, pI 7.85 and 7.7 showed 50% inhibition by leupeptin, thus they may share some properties of a cystein protease or trypsin-like serine protease. Isoforms pI 9.47 and 8.95 were completely repressed by TEW although the less basic forms were not. The PR1-like enzyme of the nematophagus fungus *V.chlamydosporium* also showed a lower sensitivity to turkey egg white than that of *M.anisopliae* PR1 (Segers et al. 1994).

Isoform pI 8.95 was not induced by starvation alone and was repressed in the presence of chitin. It was, however, produced on BSA. In fact, all isoforms produced on cuticle were present on BSA. All isoforms with the exception of pI 8.95 were produced on practical grade chitin, but none of them were recorded in GlcNAg restricted feed cultures. The common denominator appears to be non-specific protein induction.

KV42, in addition to pI ≥9.47, produced two couplets of protease with activity against the chymoelastase substrate. The first of these had pIs of 8.98 and 8.92, high activity against the PR1-like substrate and some activity against the PR2-like substrate. The second had pIs of 8.19 and 8.00, relatively low activity against the PR1-like substrate and did not hydrolyse the PR2-like substrate. All forms showed properties consistent with a chymotrypsin. In common with cysteine proteases, pI 8.0 was repressed by chymostatin, sensitive to leupeptin and was not repressed by TEW. All isoforms were

produced on insect cuticle, even when more accessible sources of carbon *or* nitrogen were supplied; all forms were repressed when the two were supplied together. With respect to carbon and nitrogen de/repression, all isoforms of PR1-like enzyme produced by KV42 are co-ordinately regulated.

Isoforms of the PR1-like protease from 3 isolates of *V.lecanii* were induced by chitin, insect cuticular protein or non-specific protein. Therefore, perhaps it is not surprising that no differences were found in the production of PR1 isoforms by Mycotal and Vertalec on locust and aphid cuticles. Both cuticles provide a source of all identified inducers, assuming no specific induction by aphid cuticular components.

Interestingly, Vertalec, produced two acid proteases on aphid cuticle, that were not apparent on locust cuticle. These proteases showed no PR1 or PR2-like activity. It seems likely that they were induced by some component(s) specific to host aphid cuticle. They may be aspartyl proteases, similar to those found by St Leger et al (1998) in cultures of *M.anisopliae*. Binding of PR1 to cuticle is a prerequisite for activity (St Leger et al. 1986d). The positively charged enzyme, therefore, can only hydrolyse negatively charged proteins. Cuticles which have a preponderance of positively charged proteins would presumably require the attentions of a negatively charged enzyme, like the acidic proteases identified in Vertalec. Unfortunately, the composition of aphid cuticle is largely unknown, so the adaptive significance of the Vertalec enzymes remains to be discovered.

Host (aphid) cuticle does not induce more PR1-like activity than non-host (locust) cuticle. However, though proteases from isolates of *V.lecanii* were able to release

protein from aphid and locust cuticle, ex-aphid isolates, Vertalec and KV42, digested 3.5 fold more host cuticle than non-host cuticle, while Mycotal, ex whitefly, did not show any differences in the extent of digestion of aphid and locust cuticles. The results were achieved using filtrates from locust cuticle cultures which contained the full complement of cuticle-degrading proteases (minus the acidic forms). The contribution of individual isoforms of PR1 to cuticle degradation was also assessed.

Interestingly, the chymoelastase isoform pI 9.10 of Vertalec, specifically induced by the cuticle, appeared to be the most efficient digestor of locust and aphid cuticle. pI 9.47 was also comparatively efficient at releasing protein from both cuticles. Isoforms pI 8.83 and 8.62 were poor at digesting locust cuticle, but could release protein far more efficiently from aphid cuticle.

For KV42, the amount of protein released from aphid cuticle by isoforms, pI $\geq$9.47, 8.98 and 8.92 was considerably greater than on locust cuticle. The two least basic forms pI 8.19 and 8.0 showed no differences in digestion of the two cuticles when similar enzyme activities were supplied to the reaction mixture. When the same amount of protein was supplied, because of low specific activity of the enzymes, against the PR1-like substrate, digestion was negligible. Thus it would appear that isoforms of PR1-like enzyme produced by different isolates of *V.lecanii* can digest and release nutrients more efficiently from host than non-host cuticle.

A different situation exists for Mycotal. All isoforms were able to digest both types of cuticle. Isoforms pI 9.47 and 8.19 digested both cuticles with equal facility. Isoforms pI 8.95 and 7.85 had greater activity against locust cuticle. This experiment should be

repeated on scale insect cuticle to confirm whether or not digestive ability of protease isoforms relates to host specificity.

The different digestive efficiencies against aphid and locust cuticle, of isoforms of PR1 produced by these isolates may reflect their ability to bind to the cuticle prior to digestion. St Leger et al (1991d) proposed that the efficiency of PR1 was determined primarily by its binding constant K_m rather than V_{max}, indicating that differences in binding efficiency determine proteolytic efficiency. Thus, differences in the basic nature of these enzymes and the number of negatively charged PR1 binding sites could affect the isoform's ability to digest the two types of cuticle. In addition to electrostatic adsorption, other factors such as the location of the protein in the protein/chitin matrix, protein-chitin interactions and protein glycosylation may also dictate the susceptibility of cuticular proteins to attack (Bidochka and Khachatourians 1994a,b).

The physical attributes of the cuticle such as hardness and chitin content, in addition to nutrient availability may all affect enzyme-mediated penetration by the fungus. Proteins in soft cuticles contain higher proportions of chitin than hard cuticles (Neville 1984) thus it is possible that proteases induced by chitin are more important in degrading the softer cuticles. Furthermore, the distributions of amino acids within cuticles differ - for example, hard cuticles are rich in alanine, whereas soft or flexible cuticles are enriched in glycine (Andersen et al. 1986). Proteins in soft cuticles tend to be hydrophilic with acidic isoelectric points; such proteins appear to be lacking in hard cuticles (Bidochka et al.1997). Furthermore, alanine and phenylalanine are the most preferred and proline the least preferred amino acids at the S1 subsite for *M.anisopliae*

PR1 cleavage (St Leger et al.1987c). Thus the composition of different cuticles could influence cuticle-degrading enzyme involvement in penetration (Bidochka et al.1997). Knowledge of the chemistry of the aphid cuticle is limited to lipid composition and the impact of honey dew contamination (Brey et a. 1985). A more detailed comparison between different host insect cuticles needs to be made if entomopathogen specificity is to be understood. Furthermore, PR1-like enzymes should not be considered in isolation from other enzymes and factors. *In vivo,* isoforms of PR1 and PR2 are likely to form just part of a pathogen's armoury that facilitates host penetration (St Leger et al. 1996b).

In addition to metabolite and substrate regulation, environmental factors, such as pH, may also control enzyme production. St Leger et al (1998) found that *Metarhizium* proteases were only synthesised at their pH activity optima, regardless of the presence of an inductive substrate, while *pr1* and *pr2* genes were actually turned off under acidic conditions. They surmised that the pH of the host cuticle may be particularly important in regulating fungal enzymes during invasion. In the present work, *V.lecanii* appeared stressed when transferred into media of low pH; the walls of some hyphal bodies appeared to be 'unhealthy' and the fungus actually released significant levels of PR1 (results not shown). Further work is needed to confirm these observations.

Fungal depolymerases can also be developmentally regulated. Valadares-Inglis and Peberdy (1997), for example, found that chitinolytic enzymes from protoplasts but not from mycelium were inducible by solubilised chitin. In the present work, significantly lower levels of PR1 were produced in blastospore forming cultures than in those comprising mycelia and hyphal fragments.

The results in this chapter suggest that there is a considerable degree of isolate variability within *V.lecanii* with respect to protease production, in terms of production, substrate specificity and regulation of isoforms. Gupta et al. (1992) also found considerable isolate variability within *B.bassiana* with respect to chymoelastase production on glucose and gelatin. Such studies highlight the limitations of single strain studies in making conclusions about virulence strategies of different fungal species.

The work in this chapter has highlighted a number of different production and regulatory strategies with respect to cuticle-degrading proteinase production by different isolates of *V.lecanii*. The ultimate aims of this research programme are to identify isolates with contrasting virulence strategies that could be combined within a mixture for improved rate and efficiency of kill. A mixture of a high proteinase producer such as Mycotal with an early producer such as KV54 may accelerate host penetration. Given the contrasting regulatory strategies of each strain, i.e. PR1-like enzymes of Mycotal and KV54 induced by protein and chitin components of the cuticles respectively, a mixture of these isolates may result in protease production over a longer period during the penetration process. Mycotal PR1-like enzymes could be expressed very early on when protein is most accessible, while the KV54 enzymes could be produced as chitin becomes available and N-acetyl glucosamine is released. If the ability of isoforms of PR1 to digest different types of cuticle contributes to host specificity, this mixture could affect the target host range of the product, allowing whiteflies and aphids to be controlled with one application.

The ability to produce isoforms of a cuticle degrading proteinase not subject to catabolite repression, readily induced by host cuticle and efficient at binding to and degrading host cuticle, would be a highly desirable trait for any strain improvement programme. It may be of use to combine an isolate such as KV42 whose proteases are not repressed by low molecular weight carbon and an isolate such as Vertalec induced to produce PR1-like enzymes by both the proteinaceous and chitin components of the cuticle. Both of these isolates exhibit a similar LD_{50} and LT_{50} to *Myzus persicae* thus it is possible that Vertalec compensates in some way for its' enzyme's sensitivity to catabolite repression. For example, isolate KV42 may enter the host earlier, and produce PR1-like enzymes in the haemolymph earlier than Vertalec, however the latter isolate may grow more profusely in the aphid haemolymph than KV42.

In vitro studies only enable us to speculate on the mode of infection of a fungal isolate. Chapter 4 addresses the production of PR1-like enzymes and fungal growth within the aphid itself to see how the infective strategies of two apparently contrasting isolates, KV42 and Vertalec, differ from one another.

4 *In vivo* studies of production of PR1-like enzymes

4.0 Introduction

In vitro production of cuticle degrading enzymes by Deuteromycete and Ascomycete entomopathogenic fungi is documented by extensive literature. The widespread, consistent occurrence, in particular, of 2 classes of cuticle-degrading protease (PR1 and PR2) implies an essential function (St Leger et al. 1986b, 1987b,c). However, demonstration of *in vivo* production is necessary to define a parasitic, as opposed to a purely saprophytic, role.

PR1 and PR2 have been immunolocalised in cuticle during infection, predominantly to the immediate vicinity of the penetrant structures (Goettel et al. 1989, St Leger et al. 1996b respectively), though both enzymes diffused throughout the cuticle during later stages of pathogenesis (Goettel et al. 1989). In contrast, Schreiter et al. (1994) found cuticle degrading protease activity along the length of the actively growing hyphae of *V.lecanii* as it invaded thrips, resulting in numerous lesions in the host cuticle. This strategy, in itself, could release enough nutrients for growth, without having to colonise the haemocoel. The immunolocalisation experiments of St Leger et al. (1996b) were performed on excised pieces of cuticle, the fate of trypsins within the host haemolymph was not investigated. These dead sections of integument may be different from mycosed whole insects in which host defence and other aspects of signal exchange can play a part. Goettal et al. (1989) found PR1 present around fungal

structures penetrating dead and living cuticle of whole insects, however the cuticle was sectioned separately from the rest of the insect for immunostaining, thus the presence of PR1 within the haemolymph was, again, not investigated.

PR1 is the major protein produced by *M.anisopliae* during penetration of the insect cuticle (St Leger et al. 1989b, St Leger et al. 1995b) and it is more efficient at degrading the cuticle than the trypsin PR2 (St Leger 1995). However, it is likely that the two enzymes work synergistically to degrade cuticle. PR2 may hydrolyse the hydrophilic groups on the periphery of globular proteins, thereby opening them up for PR1 (St Leger et al. 1996b). *In vitro* studies on the regulation of these proteinases produced by *V.lecanii* in chapter 3, revealed differences between isolates and have been discussed primarily in light of their role in cuticle penetration. St Leger et al. (1989b) found that catabolite repression in culture, also prevailed on the surface of the insect; readily available nutrients on the surface of the host delayed PR1 production.

Proteases may have additional roles to penetration during pathogenesis. The trypsins, in addition to assisting in host penetration and nutrient acquisition, could destroy host anti-fungal proteins and elevate pH by releasing amino acids for amine production (St Leger et al. 1996b). PR1-like enzymes may be insecticidal (St Leger et al. 1996c). These additional effects of fungal proteases could only occur if the enzymes are expressed inside the insect as well as in the cuticle. Some fungal exoenzymes important for nutrient acquisition, such as trehalases, α-glucosidases and acid phosphatases, are produced within the host haemolymph (Cobb, 1997), however, available evidence suggests that in the main proteinases are not (Gillespie, 1995, St Leger et al. 1996c). PR1 has not been detected during the early colonisation of

haemolymph (Gillespie, 1995), in fact wild type isolates of *M.anisopliae* do not produce PR1 again until very late in infection. To date, it is not clear whether or not the nutrient rich environment of the haemolymph is a reason for the absence of protease activity produced by the invading fungus observed by the above authors (Bidochka et al. 1997), however given that isolates of *V.lecanii* have exhibited variable sensitivities to catabolite repression, further differences may be observed between isolates later during infection.

If PR1 was produced within the insect haemolymph, it could have an insecticidal effect. Insertion of multiple copies of the *pr1* gene into the genome of *M.anisopliae* and the resultant overexpression of the enzyme in host haemolymph resulted in a reduced time to death and rapid reduction in food consumption (St Leger et al 1996c). These symptoms were thought to be due to the toxic effects of constitutive production of PR1 within the haemolymph, and activation of the phenoloxidase (PO) cascade by the enzyme - the resultant reaction products (melanin) may also be toxic to the host. Insect phenoloxidases are implicated in wound healing and defence reactions against pathogens (Gillespie et al. 1991). Phenoloxidase levels are reduced later in infection, concurrent with increased levels of PR1 in the haemolymph. Since phenoloxidases are resistant to proteolysis, it is unlikely that PR1 is involved in suppression of phenoloxidase activity (St Leger et al. 1996c).

Little is known of the toxic effects of fungal proteinases on insect haemolymph, however proteinases from entomopathogenic bacteria specifically precipitate haemolymph proteins and may also damage the fat body (Lysenko 1985). Recently, Griesch and Vilcinskas (1998) showed that proteases from *B.bassiana* impaired

phagocyte activity, attachment and spreading of plasmatocytes from the greater wax moth, *Galleria melonella in vitro.*

Host protease inhibitors regulate metabolic processes and prevent unrestrained proteolytic activity consequent on wounding or invading pathogens (Boucias and Pendland 1987). However, in addition, several studies have shown induction during mycosis of inhibitors of fungal proteases, for example inducible protease inhibitors have been detected within the haemolymph of *Anticarsia gemmatalis* (Boucias and Pendland 1987) *Galleria mellonella* (Vilcinskas and Wedde 1997) *Schistocerca gregaria* and *Manduca sexta* (Gillespie, 1995) Their existence implies a role for proteases during disease development. The invading pathogen may, however, develop specific strategies to overcome the host's defences. An increased titre of protease inhibitors occurred in *Galleria mellonella* larvae after injection with dead and living blastospores of *B.bassiana,* but not following topical application of conidia. Allowing the fungus to penetrate the cuticle naturally somehow prevented the induction of protease inhibitors (Vilcinskas and Wedde 1997). Interestingly, protease inhibitors from cabbage foliage were antifungal to two non-crucifer pathogens *in vitro* but had no effect on a fungus that specifically attacks cabbage (Lorito et al. 1994). Clearly, the ability to overcome individual protease inhibitors could contribute to host specificity.

If the regulatory mechanisms of proteases identified *in vitro* operate *in vivo*, then an isolate like KV42 capable of producing PR1 even in the presence of low molecular weight carbon or nitrogen may produce proteases very early on during mycosis, whereas the catabolite sensitive isolate, Vertalec, would produce proteases much later. In this chapter PR1-like enzymes within KV42, Vertalec and control infected aphids

were investigated by means of direct assay and immunolocalisation experiments. The results are discussed with respect to the possible role of catabolite repression in controlling PR1 production within insect haemolymph.

4.1 RESULTS

4.1.1 PR1-like activity in infected aphids over time - a comparison of KV42, Vertalec and Control Infected Aphids

Proteinase activity against the chymotrypsin substrate was detected in homogenates from non-infected insects over the six day experimental period (figure 4.1). Vertalec infected insects had similar levels of proteinase activity to controls until death of the insects (between 4 and 5 days), when there was a marked increase in activity as the fungus emerged from the host and colonised the cuticle. In contrast, KV42 infected aphids contained significantly higher levels of proteinase activity than controls or insects infected with Vertalec over the course of infection, particularly on days 2 and 3. Following death of the insect (4 -5 days), activity was high for both isolates of *V.lecanii*, but once the fungus emerged from the insect (day 6), PR1-like activity from homogenates of Vertalec infected aphids was significantly higher than that from KV42 infected insects. In this experiment, no significant difference in time to death of the host was observed for either isolate of *Verticillium lecanii* and both isolates killed over 50% of the aphid population between 4 and 5 days.

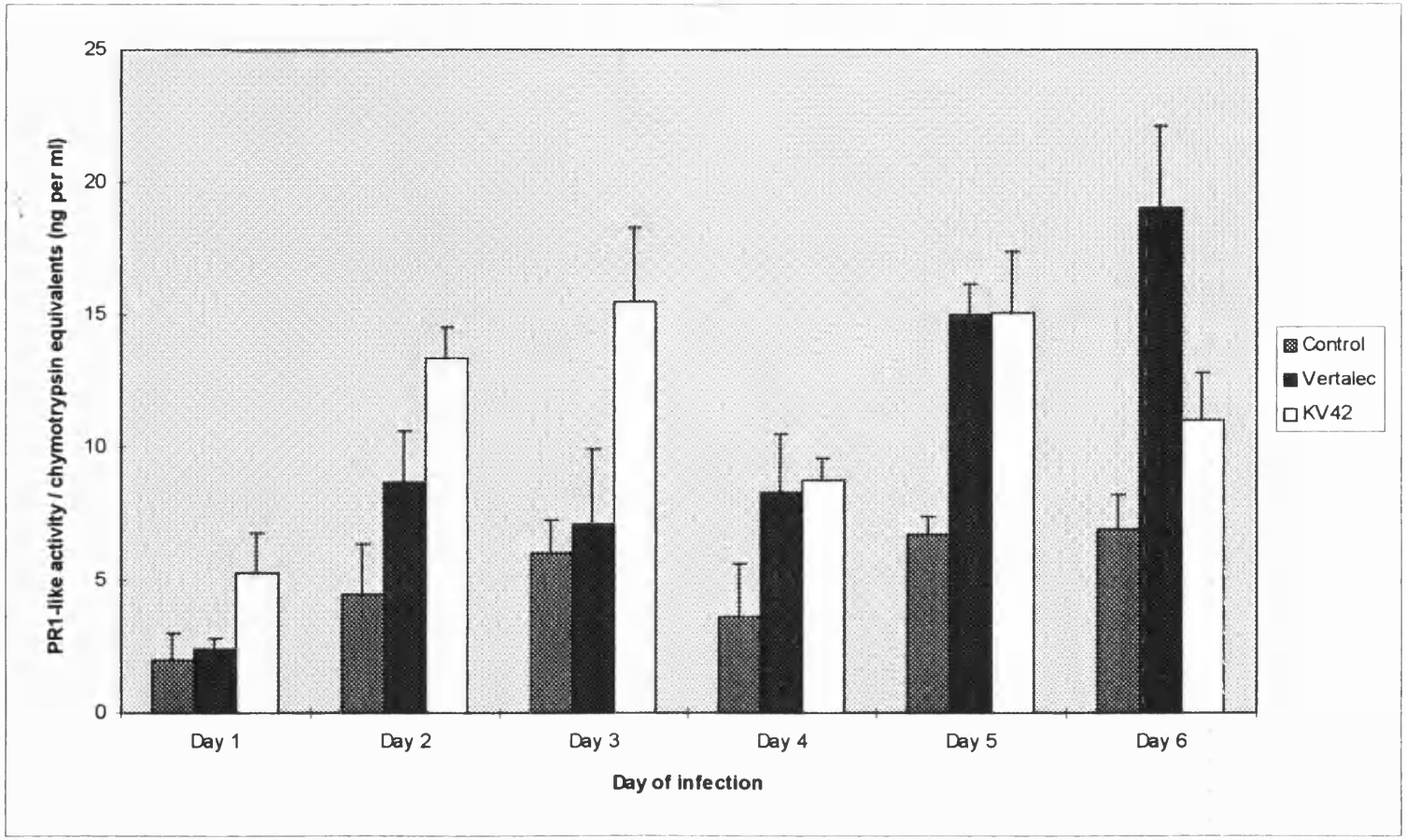


Figure 4.1. Graph showing PR1-like activity in infected and control aphids over a 6 day period. Aphids were sprayed with either sterile distilled water or 4 x 10^6 conidia per ml of *V.lecanii* (Vertalec or KV42). Aphids were collected at each day of infection and 5 aphids from each replicate were homogenised in Tris-HCl buffer (75 ± 10 μg protein per replicate) and assayed for PR1-like activity by measuring the release of amino methyl coumarin from the substrate (N-Suc-Ala-Ala-Pro-Phe-AMC) over 1 hour using a fluorescent plate reader. Mean activity is expressed as ng ml^{-1} equivalents of chymotrypsin (as estimated from known dilutions of chymotrypsin), standard deviations are shown (n=5).

4.1.2 Detection of isoforms of proteases in infected and non infected aphids

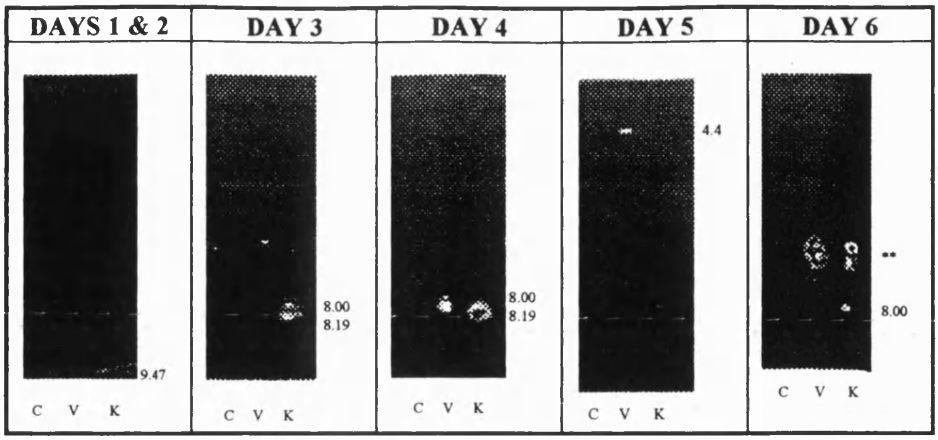


Figure 4.2. Gelatin overlay of haemolymph proteins from infected and non-infected aphids, separated by iso-electric focusing. Aphids infected with *V.lecanii*, strains KV42 (K)or Vertalec (V), or controls (C) were homogenised and ~75µg protein was loaded onto a broad range iso-electric focusing gel. After focusing, the gel was overlaid with gelatin film, and the position of proteases noted after incubation overnight in a damp chamber. ** indicates region of application piece, whereby sample was loaded (pI 6.8-7.5).

The protease activity in each sample was very low, as no degradation of the overlay was noted until 24 - 36 hours. Gel slice assays were also done and some hydrolysis of the chymotrypsin substrate was observed with slices at pI ≥9.5, 8, 8.19 and at the region of loading of the sample. However activity was too low to make quantitative comparisons.

A very basic protease (pI ≥ 9,.47) was detected in aphids of all treatments over the course of the experiment as indicated by gel slice assays, although only during the first 2 days of the experiment was it detectable by gelatin overlay. Its appearance in control insects indicates that it must be produced by the insect. A number of other isoforms were detected only in mycosed insects. Two proteinases with pIs of 8.00 and 8.19 were found in extracts from KV42 infected insects on days 3 and 4. A proteinase of pI 8.00 was also detected in Vertalec infected insects on day 4 (as insects were beginning

to die). Additional proteolytic activity was detected at the point of loading on the IEF gel (pI 6.8-7.5) with both isolates on day 5 and 6 (post-mortem, when the fungus was emerging from the cadavers). Finally, an isoform of protease of pI 4.4 was found in aphids infected with Vertalec, this did not hydrolyse the chymoelastase substrate.

Summary:

Estimated pI of protease			
Day of infection	Control	Vertalec	KV42
1	≥9.5	≥9.5	≥9.5
2	≥9.5	≥9.5	≥9.5
3	≥9.5	≥9.5	≥9.5 8.19 8.00
4	≥9.5	≥9.5 8.00	≥9.5 8.19 8.00
5	≥9.5	≥9.5 ** 4.4	≥9.5 **
6	≥9.5	≥9.5 **	≥9.5 **

** protease activity detected at the point of sample loading (pI 6.85-7.35)

4.1.3 Localisation of PR1-like enzymes within the host using polyclonal antibodies

In order to provide further evidence for production of PR1-like enzymes by *Verticillium lecanii* within infected aphids, polyclonal antibodies were raised to a purified enzyme from each strain, which were then used in immunolocalisation experiments.

4.1.3.1 Purification of PR1-like enzymes

For both isolates of *V.lecanii*, KV42 and Vertalec, the isoform used for generating antibodies, was the most basic form, pI $\geq$9.47. This was chosen because it could be separated easily from other proteins on the iso-electric focusing gel, even when high levels of protein were applied to the gel. In addition, it was the only form to be produced by both isolates and it was highly active against the PR1 substrate. Levels of purification of the enzyme from each isolate are summarised in figure 4.3.

For each isolate, PR1-like activity was induced by transfer of a starved 4 d old biomass of fungus into basal salts containing 1% insect cuticle. Similar high specific activities were recorded for each isolate of *V.lecanii*. A total protein precipitation using 70% ammonium sulphate resulted in a 3.7 fold and 4.4 fold purification for Vertalec and KV42 respectively. PR1-like activity appeared to remain stable at all dialysis stages. Preparative isoelectric focusing using the Rotofor was not essential, however, it did provide a 53.8 and 37.1 fold purification level for Vertalec and KV42 respectively, and it enabled improved yields of purified enzyme in the next stage. A good level of

purification was obtained through flat bed iso-electric focusing of Vertalec protease (~110) and KV42 (~78 fold).

Figure 4.3. Purification of an extracellular chymoelastase from cuticle cultures of *Verticillium lecanii* 24 h post transfer. *

a) Vertalec

Purification Stage	Specific activity (nkat mg^{-1})	Purification (fold)
i) Crude Filtrate	44	1.00
ii) 70% Ammonium sulphate precipitation	161	3.7
iii) Preparative IEF	2369	53.8
iv) Flat bed IEF	4872	110.7

b) KV42

Purification Stage	Specific activity (nkat mg^{-1})	Purification (fold)
i) Crude Filtrate	50.5	1.00
ii) 70% Ammonium sulphate precipitation	220	4.4
iii) Preparative IEF	1875	37.1
iv) Flat bed IEF	3971	78.6

* Enzyme was purified from cuticle cultures inoculated with established biomass, data represent typical run.

The Vertalec proteinase was successfully purified to a single band of 30.5 kDa as viewed by Coomassie staining of an SDS-PAGE gel (Figure 4.4a). No further bands were observed with silver staining (not shown). KV42 was purified partially to a band

of 31.8kDa as viewed by Coomassie staining, although contaminating bands were also noted at 41.5 and 43kDa (4.4b). For the purposes of excision and injection into rabbits to raise polyclonal antibodies the 31.8kDa band could be excised cleanly away from the contaminating proteins.

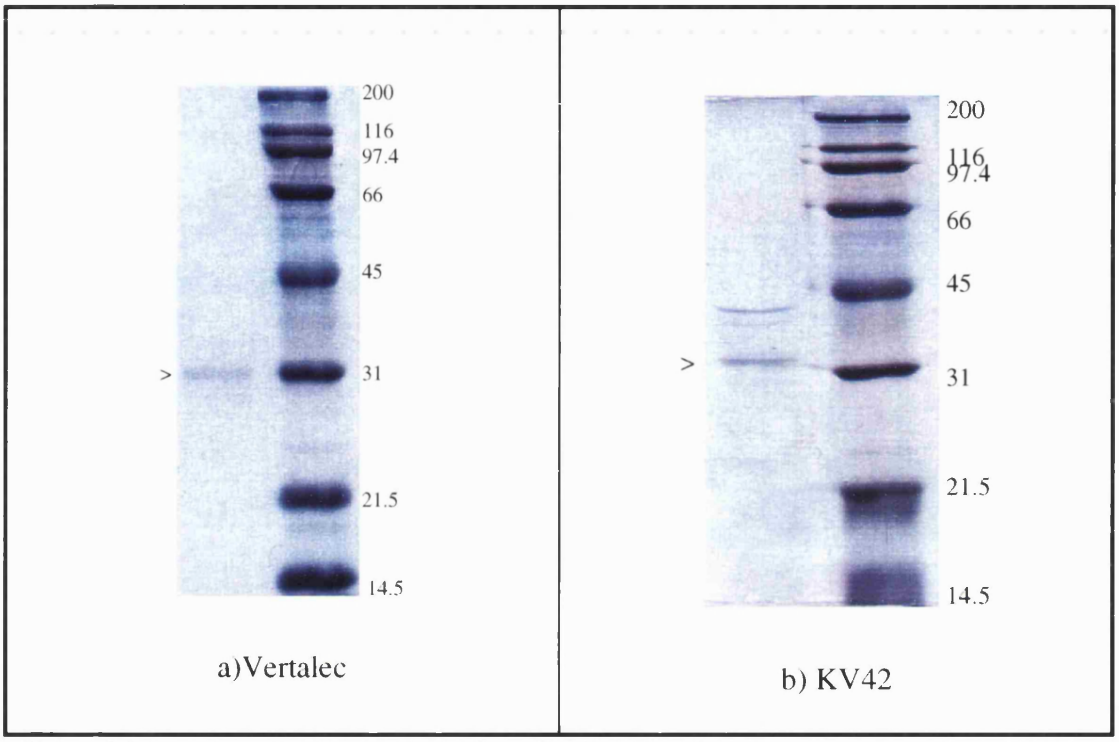


Figure 4.4 Coomassie stained gel of purified/semi-purified PR1-like enzyme from Vertalec and KV42 cuticle cultures respectively. Protein marked for excision from the gel and for subsequent boosts of rabbits to raise polyclonal antibodies to the enzyme.

A number of lines of evidence suggest that the bands depicted are PR1-like enzymes:-

1. Both enzymes had a high specific activity to the substrate N-Suc-Ala-Ala-Pro-Phe-pNA, prior to loading onto SDS-PAGE.

2. For Vertalec only one band could be visualised with Coomassie Blue and Silver Stain. Although 2 contaminating bands (>40kDa) were observed in KV42 cultures, a band similar in size to Vertalec (~31kDa) was observed.

3. When the samples were run on native-PAGE, and tested with a gelatin overlay, protease activity was detected only at the intersection between the stacking and resolving gels. Staining the gel with Coomassie blue and silver stain, revealed that the band at 31kDa for each isolate was absent, but a new band at the intersection of the two gels was present. 2 bands at ~40kDa could still be visualised for KV42 and did not possess any protease activity. It is possible that the basic nature of the protease precluded its entry into the gel at the charge of the buffer used in these native conditions.

4. Rotofor fractions positive for PR1-like activity, contained bands at ~31kDa, those with no detectable activity i.e. at low pH, did not.

4.1.3.2 Titre and Specificity of Polyclonal Antibodies

i) Estimation of Antibody Titre

Rabbits were boosted with 30-60μg pure protein from Vertalec and KV42, as excised from an SDS-PAGE gel every 14 days until antibody titre was deemed to be high enough. Blood samples were taken 1 week after each boost and were tested in a 'dot

blot' system against crude and pure protein to estimate the progress of antibody production over time (figures 4.5 and 4.6). 2 rabbits for each isolate were used.

Figure 4.5. Dot blots of pure and crude protein from Vertalec cultures tested with serum from 2 rabbits at each stage of boosting.

Figure 4.6. Dot blots of pure and crude protein from KV42 cultures tested with serum from 2 rabbits at each stage of boosting.

20 µg crude filtrate from cuticle cultures (a) and 5 µg pure PR1-like enzyme (b) were applied to nitrocellulose and incubated with serum extracted from each rabbit before boosting commenced (pre-bleed) and at each test stage at 3 dilutions (1:100, 1:500 and 1:1000) in Tris-buffered saline. The blot was developed using an anti-rabbit secondary antibody bound to horse-radish peroxidase and developed using hydrogen peroxide and chloro-1-napthol.

Vertalec Boosted (Rabbit 217)

Dilution	PRE-BLEED	1ST TEST	2ND TEST	3RD TEST	4TH TEST	5TH TEST
1:100	a.  b. 	a. b.	a.  b.	a.  b. 	a.  b. 	a.  b. 
1:500	a. b. 	a. b.	a. b.	a. b.	a. b.	a. b.
1:1000	a. b.	a. b.	a. b.	a. b.	a. b.	a. b.

Vertalec Boosted (Rabbit 218)

Dilution	PRE-BLEED	1ST TEST	2ND TEST	3RD TEST	4TH TEST	5TH TEST
1:100	a. b. 	a. b.	a. b.	a. b.	a. b.	a. b.
1:500	a. b.	a. b.	a. b.	a. b.	a. b.	a. b.
1:1000	a. b.	a. b.	a. b.	a. b.	a. b.	a. b.

KV42 Boosted (Rabbit 221)

Dilution	PRE-BLEED	1ST TEST	2ND TEST	3RD TEST	4TH TEST	5TH TEST
1:100	a. b.	a. b.	a. b.	a. b.	a. b.	a. b.
1:500	a. b.	a. b.	a. b.	a. b.	a. b.	a. b.
1:1000	a. b.	a. b.	a. b.	a. b.	a. b.	a. b.

KV42 Boosted (Rabbit 222)

Dilution	PRE-BLEED	1ST TEST	2ND TEST	3RD TEST	4TH TEST	5TH TEST
1:100	a. b.	a. b.	a. b.	a. b.	a. b.	a. b.
1:500	a. b.	a. b.	a. b.	a. b.	a. b.	a. b.
1:1000	a. b.	a. b.	a. b.	a. b.	a. b.	a. b.

i) Vertalec

Both rabbits used responded to the boosts of antigen given. However, in each case, the pre-bleed, that is blood that had not been in contact with the antigen, was shown to bind to both crude filtrate and pure PR1-like enzyme from Vertalec. The signal was strong at 1:100 dilution of antibody, clear at 1:500 and feint at 1:1000. Serum from other non-boosted rabbits was also shown to recognise Vertalec proteins at this concentration (results not shown). For both rabbits, after the first injection of antigen, the reaction appeared to be far less than the pre-bleed, however as boosting continued, the antibody appeared to strongly bind to the antigen. For each serum, at 1:100 dilution of antibody, after the fourth boost a very strong signal was obtained for the crude filtrate and pure enzyme (the latter being much more intense). For rabbit 217, this was noticeably darker than in the pre-bleed. By using these amounts of protein, the peak antibody titre appeared to be after the 4th boost, recognising the antigen up to a dilution of 1:1000 of antibody. Boosting ceased after the fifth bleed since no further improvement in signal was observed.

ii) KV42

The two KV42 injected rabbits were also shown to respond to boosts of antigen, however the final titre of antibodies appeared to be lower than that for Vertalec boosted rabbits. Similarly, the pre-bleed recognised the pure and crude filtrate from KV42 cultures at 1:100 and 1:500 dilutions, although a weaker signal was given compared to that of pre-bleed to Vertalec proteins. For each rabbit, the response to the injection of antigen was slow, however, after 5 boosts a strong signal was obtained, the serum binding well to both crude and pure protein at 1:100 and 1:500 dilutions of

antibody. The intensity of the dots was less than that for peak reaction in Vertalec injected rabbits. The serum was able to detect the antigen at 1:1000 dilution although the signal was feint.

For subsequent tests, serum from rabbit 217, 4th bleed, and rabbit 221, 5th bleed were used.

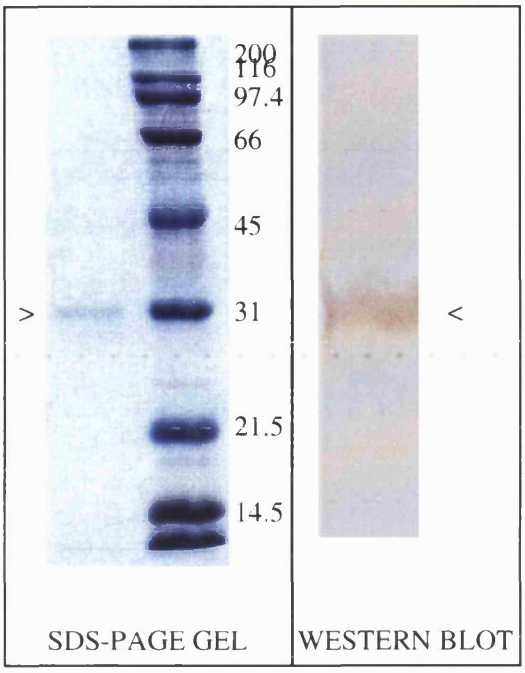
ii) Western Blotting

Western blots were performed to ensure that, the antibodies identified and bound to a blot of the semi-purified protein after SDS-PAGE (Figure 4.7) and secondly, were able to locate the enzyme from the other proteins from within crude filtrate (Figure 4.8).

A band could be seen at approximately 31kDa for both Vertalec and KV42, corresponding to that shown on the Coomassie Stain. This confirmed that the transfer conditions were suitable and that the antibodies could recognise the protein after blotting, and that background staining was minimal

For Vertalec crude filtrate, after developing the Western, a band was observed at 31kDa, corresponding to the purified PR1-like enzyme. Lower dilutions that 1:1000, did not work well. At a 1:1000 dilution of antibody, there was no cross-reaction to other bands present. Similarly, a band was observed for KV42, with a Mr slightly higher than that for Vertalec.

a) Vertalec



b) KV42

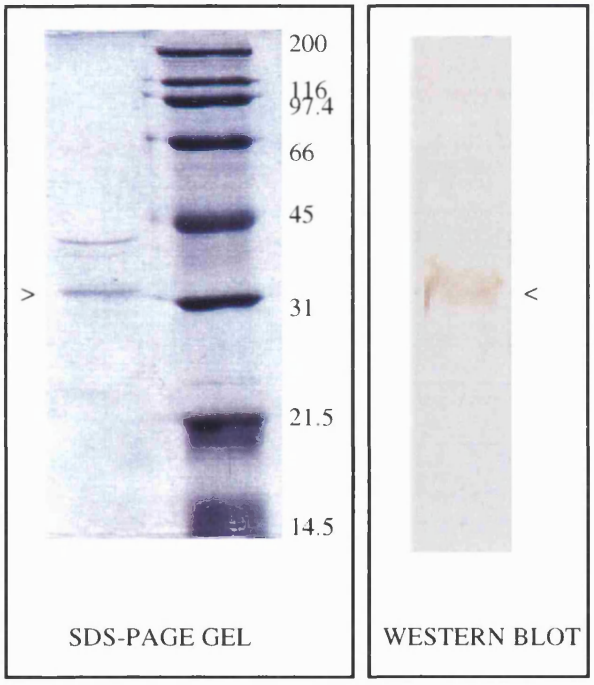
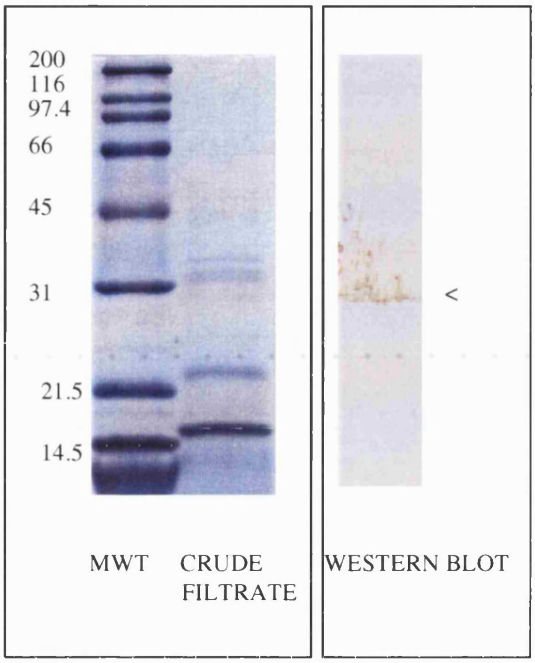


Figure 4.7 40µg of purified protein was loaded onto an SDS-PAGE gel and electrophoresis conducted for 1 hour at 150V. Half the gel was stained with Coomassie blue, and the remainder was blotted for 1 hour, 300mA and developed as described in chapter 2 using a 1:1000 dilution of antibody and the Vectastain™ ABC kit. Antibodies raised against the enzyme from Vertalec and KV42 were used.

a) Vertalec



b) KV42

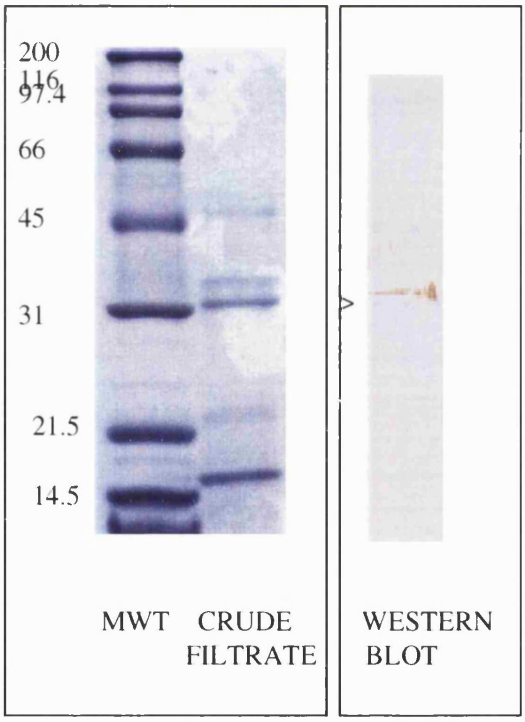


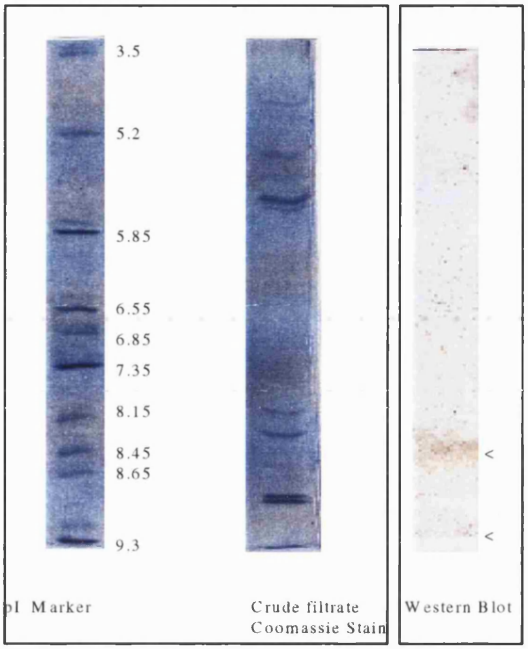
Figure 4.8. 100µg protein from concentrated crude filtrate from each strain was loaded onto an SDS-PAGE gel and electrophoresis conducted for 1 hour, 150V. Half the gel was stained with Coomassie blue, and the remainder was blotted for 1 hour, 300mA and developed as described in chapter 2 using a 1:1000 dilution of antibody and the Vectastain ABC™ kit. Antibodies raised against the Vertalec and KV42 enzymes were used as appropriate.

A Coomassie Blue stain of the gel for each isolate revealed a major protein at ~32kDa, however, during purification this was found not to be the PR1-like enzyme, in fact it seemed to 'mask' the desired protein. In each Western of crude filtrate, the bands were feint and were very slow to appear relative to that of the purified protein. Given the weakness of this reaction for crude filtrate, it was unlikely that Westerns could be performed on aphid haemolymph, thus *in vivo* studies were performed by means of immunocytochemistry (section 4.1.3.3).

Attempts were made to blot iso-electric focusing gels using an adaptation of the Southern blot method, to see if antibodies were specific for particular isoforms.

Blots of iso-electric focusing gels of crude filtrate from Vertalec and KV42 were developed using the Vectastain™ ABC kit (figure 4.9). Since the reaction was very slow, colour was allowed to develop overnight. For KV42, against the background could be seen two bands in particular, corresponding to approximately pI 9.5 and 8.20. However, for Vertalec, a strong reaction was observed at the basic end of the gel, but bands were too diffuse to discern.

a) KV42



b) Vertalec

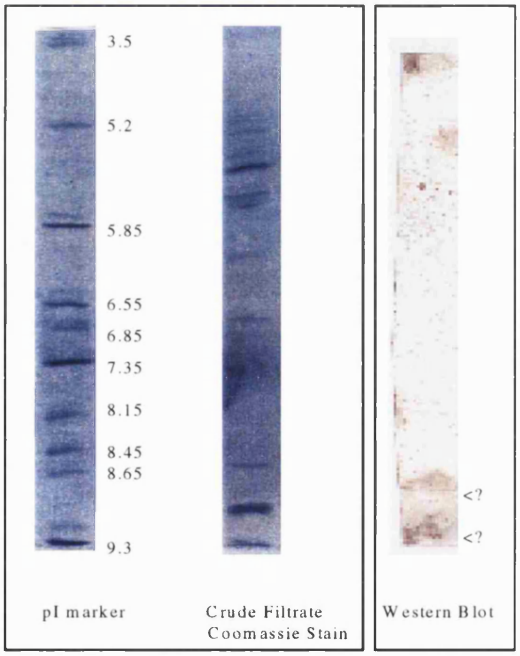


Figure 4.9 Blot of IEF gel. 100µg of protein from crude cuticle culture filtrate of each strain was focused on an IEF gel for 1.5h at a constant wattage of 30W. Half the gel was stained with Coomassie blue and the remainder was 'passively' blotted for 4 hours and then developed using a 1:1000 dilution of antibody and the Vectastain™ ABC kit.

iii) Cross-reactivity of antibodies

The ability of antibodies, raised to the purified chymoelastase from either Vertalec or KV42 to cross react with pure enzyme from the other strain, and PR1 and PR2 from *Metarhizium anisopliae* was investigated (Figure 4.10).

a) Polyclonal antibodies raised to a PR1-like enzyme from Vertalec

Dilution	Pure PR1-like enzyme from Vertalec (*V.lecanii*)	Pure PR1-like enzyme from KV42 (*V.lecanii*)	Pure PR1 from *Metarhizium anisopliae* strain ME1	Pure PR2 from *Metarhizium anisopliae* strain ME1
1:1000				
1:4000				

b) Polyclonal antibodies raised to a PR1-like enzyme from KV42

Dilution	Pure PR1-like enzyme from Vertalec (*V.lecanii*)	Pure PR1-like enzyme from KV42 (*V.lecanii*)	Pure PR1 from *Metarhizium anisopliae* strain ME1	Pure PR2 from *Metarhizium anisopliae* strain ME1
1:1000				
1:4000				

Figure 4.10 Dot blots of pure PR1-like enzyme from Vertalec, KV42 and PR1 and PR2 from *Metarhizium anisopliae*, strain ME1. 5 µg protein of each isolate was applied to nitro-cellulose and incubated with serum raised to a chymoelastase from Vertalec or KV42 at 1:1000 and 1:4000 dilution in Tris buffered saline. The blot was developed using the Vectastain™ ABC kit.

For improved sensitivity, the biotin/avidin system was used whereby the signal from the biotinylated secondary antibody was amplified, thus dilutions of antibody at 1:1000 and 1:4000 could be used. At 1:1000, antibodies raised to Vertalec PR1-like enzyme strongly bound to 5μg of the enzyme. They also appeared to recognise pure PR1 from KV42 and *M.anisopliae*, although a much lower signal was obtained for the latter. There was little or no detection of PR2 from *M.anisopliae*. At 1:4000 the intensity of the 'dot' was still high for pure Vertalec PR1-like enzyme, but was feint for that from KV42. Antibodies did not appear to recognise PR1 and PR2 from *M.anisopliae* at this concentration.

Polyclonal antibodies raised to KV42 enzyme appeared to recognise the purified chymoelastase from each strain equally well at 1:1000 dilution, however they were poor at detecting PR1 or PR2 from *M.anisopliae*. At 1:4000, no signal was observed for the 2 enzymes from *M.anisopliae* and a very weak blot was observed for Vertalec and KV42 enzymes, although again the antibody appeared to bind equally.

Crude filtrate from Vertalec and KV42 crude cultures was then tested in a dot blot system at 4 different protein amounts (20μg, 2μg, 0.2μg and 0.02μg) to check the sensitivity and cross reactivity of the antibodies at 1:1000 and 1:4000 dilution (figure 4.11) Dot blots were also performed using filtrate from *V.lecanii* (Mycotal), *Metarhizium anisopliae* (ME1), *M.flavoviride*, *Beauveria bassiana* (all grown on insect cuticle) and *Trichoderma harzianum* (aggressive strain, grown on fungal cell walls). All filtrates had substantial PR1-like protease activity.

a) Polyclonal antibodies raised to a PR1-like enzyme from Vertalec

Dilution	*V.lecanii*: KV42				*V.lecanii*: Vertalec				*V.lecanii*: Mycotal	*Metarhizium anisopliae*: ME1	*Metarhizium flavoviride*	*Beauveria bassiana*	*Trichoderma harzianum*
1:1000	a.	b.	c.	d.	a.	b.	c.	d.					
1:4000	a.	b.	c.	d.	a.	b.	c.	d.					

b) Polyclonal antibodies raised to a PR1-like enzyme from KV42

Dilution	*V.lecanii*: KV42				*V.lecanii*: Vertalec				*V.lecanii*: Mycotal	*Metarhizium anisopliae*: ME1	*Metarhizium flavoviride*	*Beauveria bassiana*	*Trichoderma harzianum*
1:1000	a.	b.	c.	d.	a.	b.	c.	d.					
1:4000	a.	b.	c.	d.	a.	b.	c.	d.					

Figure 4.11. Dot blots of crude filtrate of isolates of *V.lecanii*, *M.anisopliae*, *M.flavoviride*, *B.bassiana* and *T.harzianum*. 4 amounts of Vertalec and KV42 protein (a. 20µg; b. 2µg; c. 0.2µg and d. 0.02µg) and 20µg each of the other isolates was applied to nitrocellulose and incubated with 1:1000 and 1:4000 dilutions of antibody and developed using the Vectastain™ ABC kit as described.

At an amount of 20 μg, at 1:1000, antibodies raised to Vertalec cross reacted well with crude filtrate from Vertalec and KV42 cuticle cultures. At this same amount of protein, antibodies appeared to bind well, albeit less strongly than for KV42 and Vertalec, to *M.anisopliae, B.bassiana* and *T.harzianum.* The signal was weaker for *M.flavoviride* and poor for *V.lecanii* isolate, Mycotal. Vertalec enzyme was detected from filtrate at amounts as low as 0.2μg , KV42 only as low as 2μg.

At 1:4000, antibodies raised to Vertalec PR1, appeared to become more specific, again binding well to Vertalec filtrate at a 20 μg, but only weakly to all other strains tested.

For 1:1000 dilution of antibodies raised to KV42, cross reactivity was again observed to filtrate from both Vertalec and KV42. However it only detected 2μg protein. Cross reactivity to other strains appeared to be weak and principally to *M.anisopliae* (ME1) and *T.harzianum* (aggressive strain).

At 1:4000, there still appeared to be no preference for either filtrate, in fact, a strong signal could be discerned against Vertalec crude filtrate. Very low levels of cross reactivity could be seen against all other strains.

4.1.3.3 Immunolocalisation Experiments

Myzus persicae was sprayed with 1 x 10^7 conidia ml^{-1} of KV42 or Vertalec and control insects were inoculated with dH_20 as described in chapter 2. Aphids were removed on each day after inoculation, fixed and sectioned for light microscopy or electron microscopy as previously described.

Immunostaining was performed with 1:2000 dilutions of antibody and developed using the Vectastain™ ABC kit and sections were background stained with Toluidine blue. No immunostaining was observed when sections of control aphid were incubated with antibody raised to KV42 PR1 (plate 4.1) or Vertalec PR1.

Negative controls whereby secondary antibody was applied to infected aphid sections without incubation with primary antibody, did not exhibit any immunostaining, confirming that any reaction was due to binding of the primary antibody and not non-specific binding of the secondary antibody.

Plate 4.1 **Control aphid.** Near horizontal sections of non infected aphids were taken and incubated with antibodies raised to the PR1-like enzyme of isolate KV42 (1:2000 dilution) and the Vectastain™ ABC kit and ImmunoPure™ metal enhanced DAB substrate, as described in chapter 2. The aphid is background stained with Toluidine Blue. Key structures are labelled. Bar represents 0.1mm.

AL: Antennal lobe

OL: Optic lobe

SG: Salivary gland

B: 'Brain'

G: Gut

L: Legs

OF: Unborn Offspring

FB: Fat body/adipose tissue

C: Cuticle


AL
SG
OL
B
L
G
L
C
OF
OF
FB

Plate 4.2 Early infection of aphid by isolate KV42. Horizontal sections of aphid 1-2d post application of KV42 conidia were taken and incubated with 1:2000 dilution of antibody (raised to PR1-like enzyme of KV42) and developed with the Vectastain™ ABC kit as described. Presence of the protease is indicated by the yellow-brown (immuno) staining (IS).

a. Aphid section background stained with Toluidine Blue

b. Aphid section without background staining

(Bar represents 0.1mm)

AL: Antennal lobe

OL: Optic lobe

SG: Salivary gland

B: 'Brain'

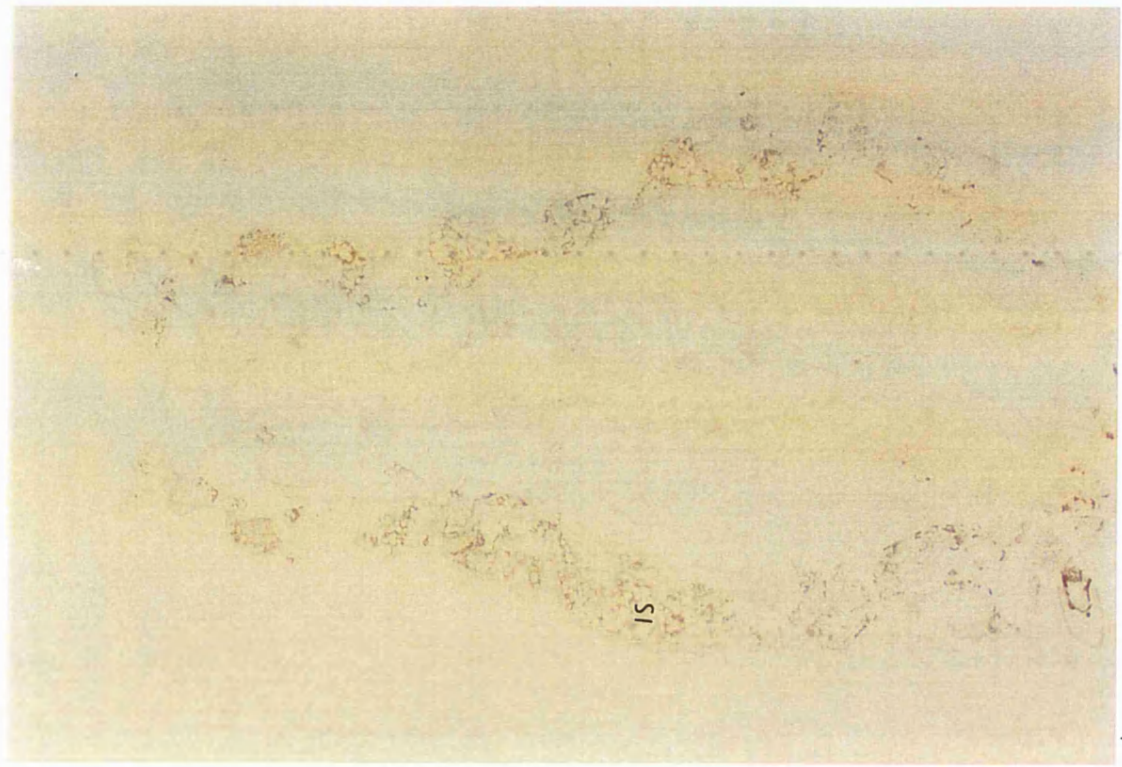
G: Gut

L: Legs

OF: Unborn Offspring

FB: Fat body/adipose tissue

C: Cuticle



b.

a.

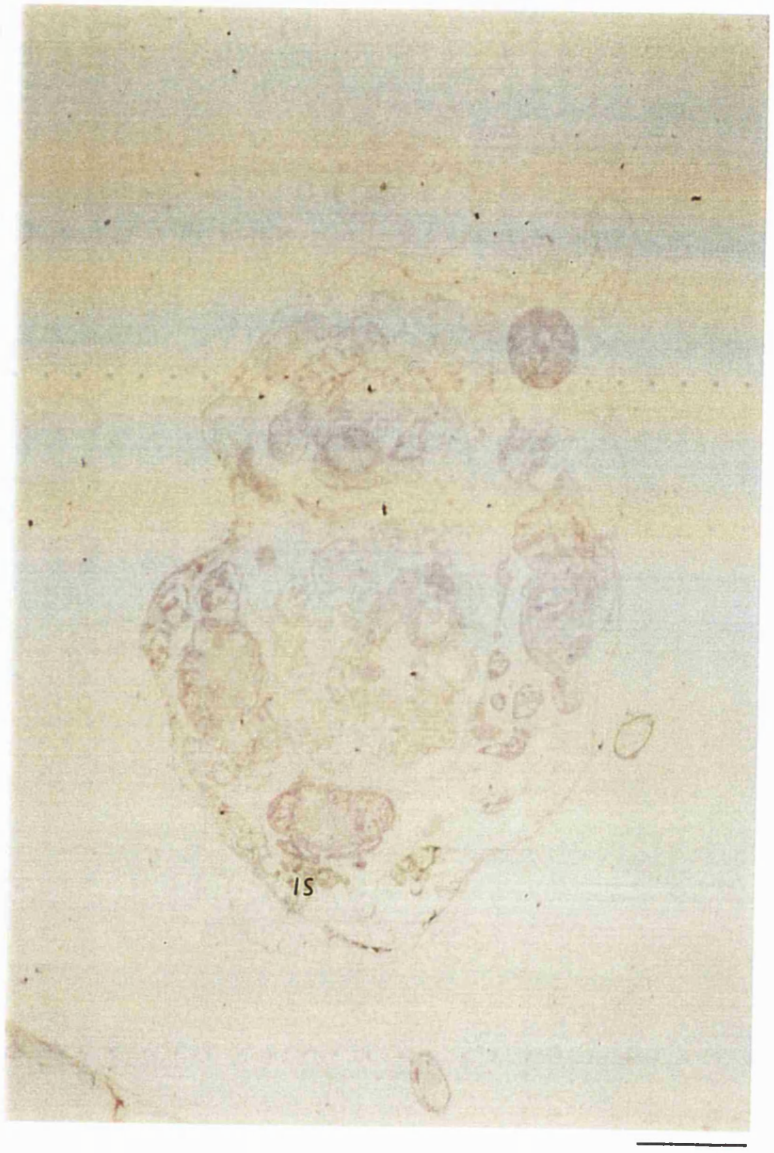


Plate 4.3 **Early infection of aphid by Vertalec.** Horizontal sections of aphid 1-2d post application of Vertalec conidia were taken and incubated with 1:2000 antibody (raised to a PR1-like enzyme of Vertalec) and developed with the Vectastain™ ABC kit as described. Section is background stained with Toluidine Blue. Presence of the protease is indicated by the yellow-brown (immuno) staining (IS).

(Bar represents 0.1mm)

In KV42 infected aphids 2d post infection, immunostaining (brown coloration) was very dense within the cuticle and around the outer tissues of the insect (plate 4.2). The enzyme was detected within the fat body of the insect, within the legs, and even the eye, however, no enzyme was detected around the unborn babies or gut area. Very little immunostaining was observed in the Vertalec infected aphid at this early stage of infection. The enzyme could only be detected in a small area to the posterior of the insect (plate 4.3).

On close inspection, high levels of enzyme were observed within the KV42 infected insect, throughout the fatty tissue around the edge of the insect (Plate 4.4). Antibodies raised to the Vertalec enzyme bound to the aphid section in an identical pattern to those raised to KV42. No fungus could be seen in the insects at this early stage of infection.

Plate 4.5 shows the enzyme to be concentrated within the adipose tissue of aphids infected with both isolates. Very little immunostaining could be seen in the Vertalec infected aphids, although fungal blastospores could be seen just within the cuticle wall.

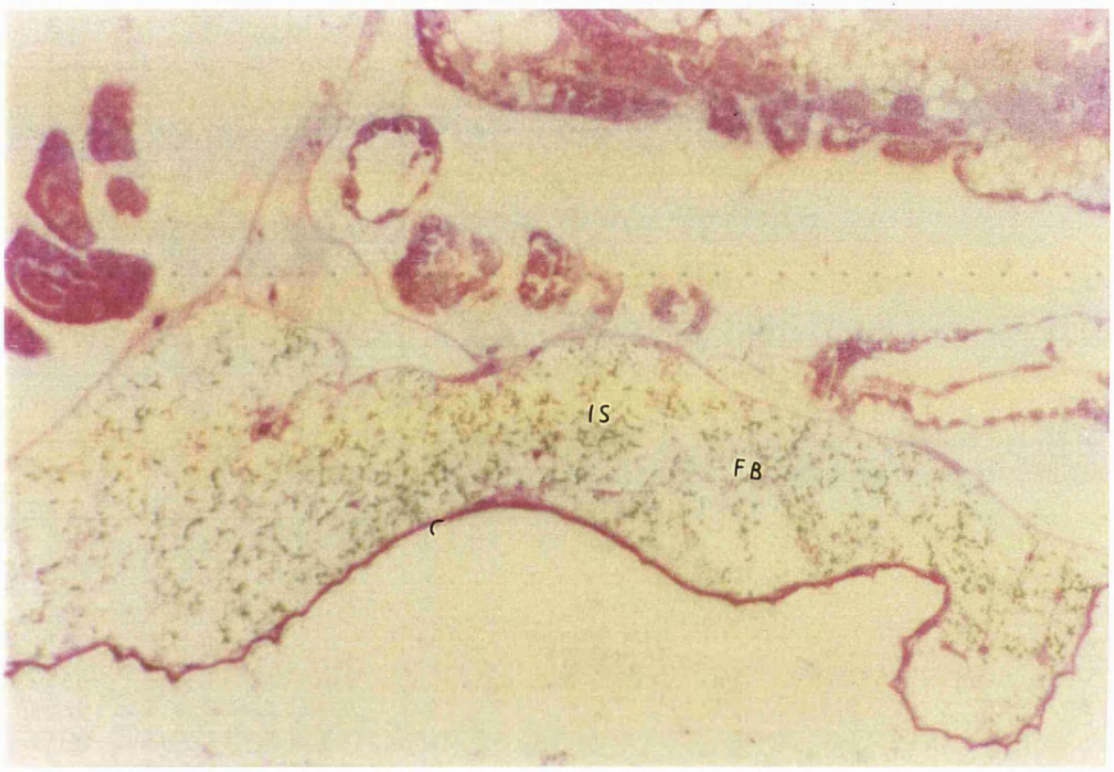
3-4d post infection, the tissue of the KV42 infected aphid still appeared to be relatively intact, although the enzyme was detected throughout the insect tissue (Plate 4.6b). In Vertalec infected insects, at this stage, large sections of insect tissue appeared to be degraded and chymoelastase enzymes could be detected throughout this degraded tissue (Plate 4.6a). A number of hyphal fragments/bodies could also be seen.

Plate 4.4 Cross Reactivity of antibodies. Horizontal sections of aphid 1-2d post application of KV42 were taken and incubated with 1:2000 dilution of antibodies raised to a PR1-like enzyme from a) KV42 and b) Vertalec, and developed as described. Presence of the protease is indicated by the brown (immuno) staining (IS).

C = Cuticle

FB = Fat Body

Bar represents 25μm.



b.

a.

Plate 4.5 Early infection of aphids with Vertalec and KV42. Horizontal sections of aphid 1-2d post application of a) KV42 conidia and b) Vertalec conidia were taken and incubated with 1:2000 dilution of antibodies raised to PR1-like enzymes from the respective strains, and developed as described. Presence of the protease is indicated by the brown (immuno) staining (IS).

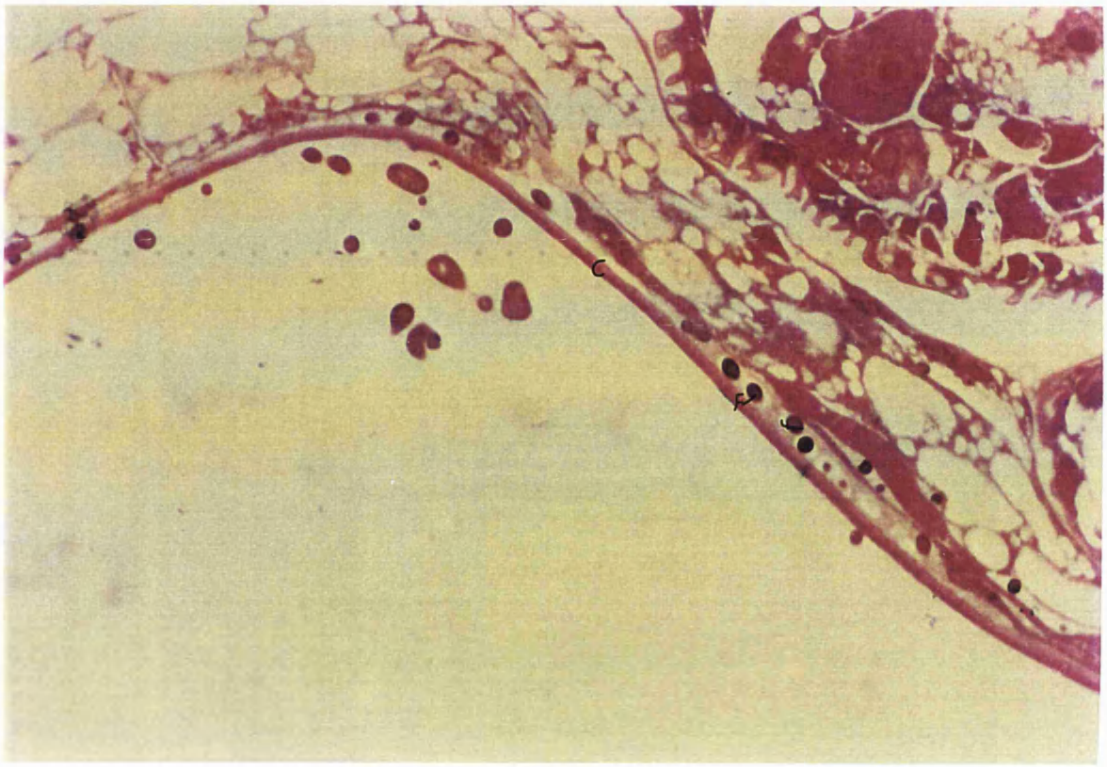
Bar represents 25μm

F = Fungal Material

C = Cuticle

FB = Fat Body

AS = Air sac


C
b.

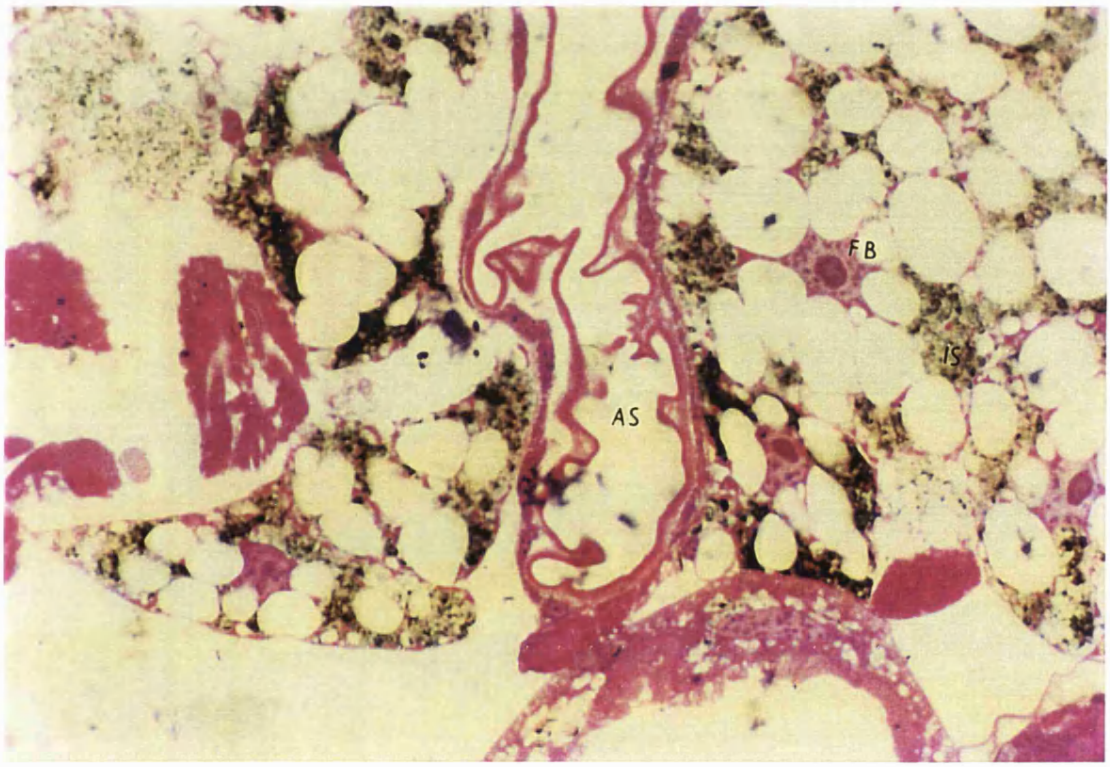

FB
IS
AS
a.

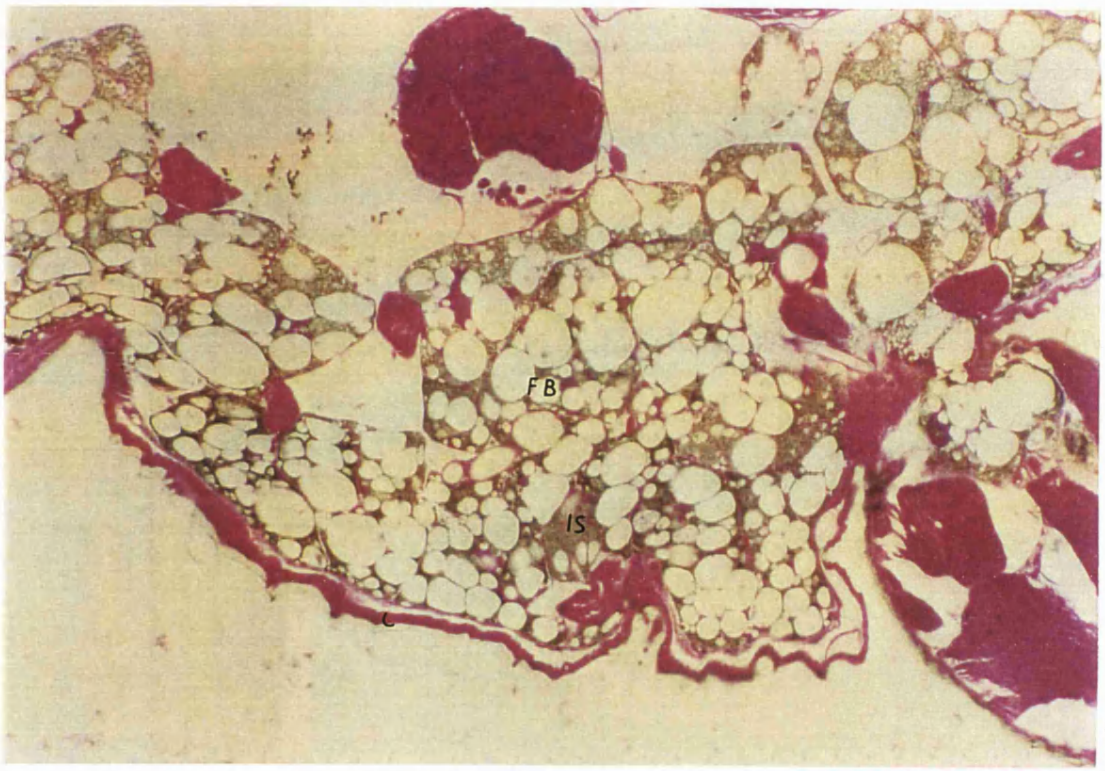
Plate 4.6. Late infection of aphids with Vertalec and KV42. Horizontal sections of aphid 3-4d post application of a) Vertalec and b) KV42 conidia were taken and incubated with the respective antibody at a 1:2000 dilution and developed as described. Presence of the protease is indicated by the golden brown (immuno) staining (IS).

Bar represents 25µm.

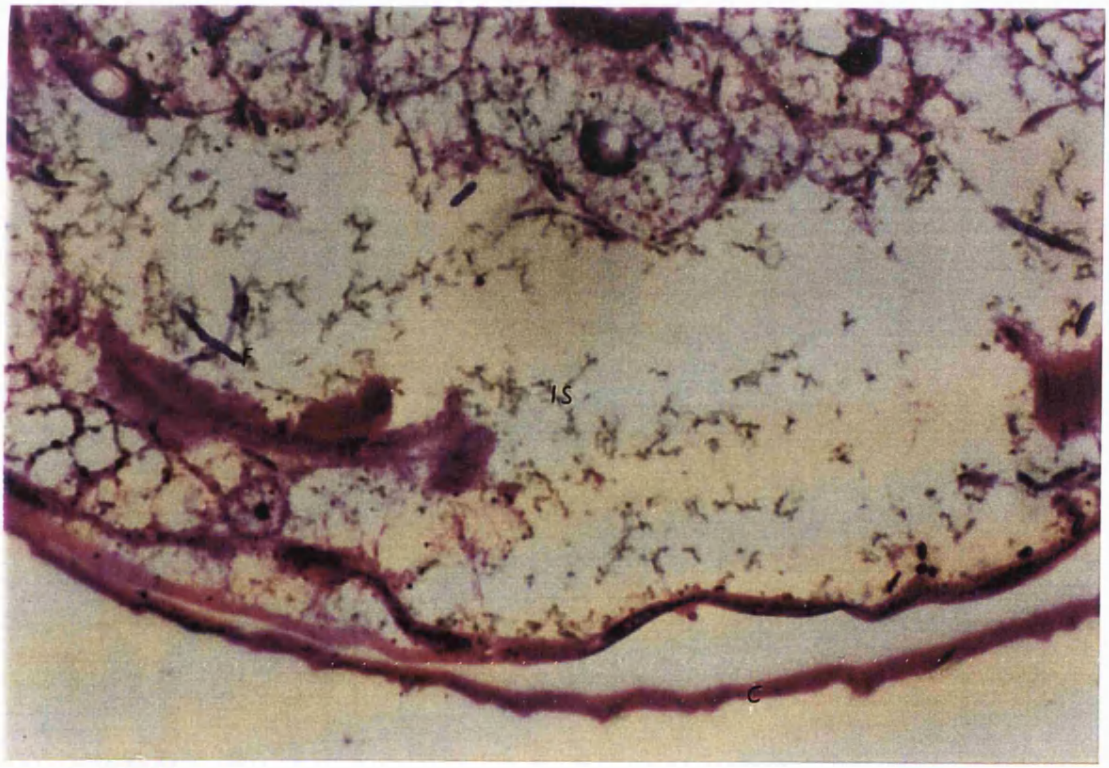
C = Cuticle

FB = Fat Body

F = Fungal Material



b.



a.

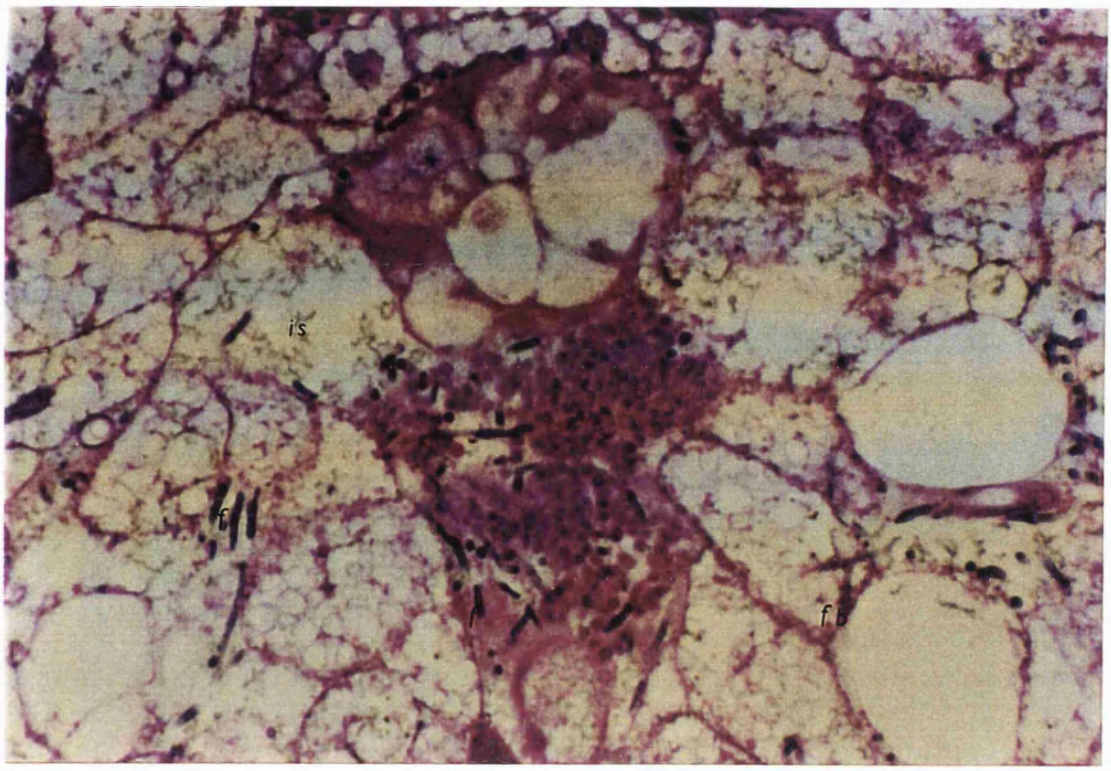
Plate 4.7. Late infection of aphid by Vertalec. Horizontal sections of aphid 4 d post application of Vertalec conidia were taken and incubated with antibody at 1:2000 dilution and developed as described. Presence of the protease is indicated by the golden brown (immuno) staining (IS).

Bar represents 10μm.

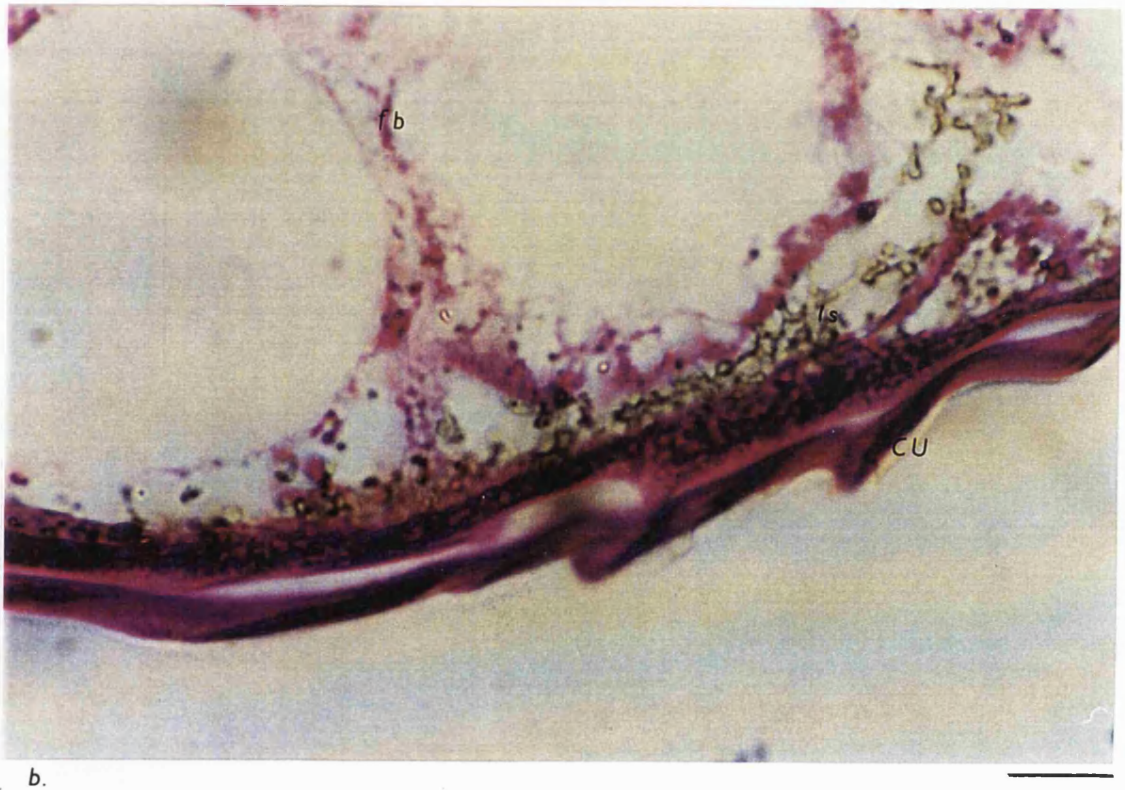
CU = Cuticle

FB = Fat Body

F = Fungal Material



a.



b.

After 4 d, Vertalec fungal material and associated chymoelastase production could be seen throughout the aphid tissue (Plate 4.7a) and on death of the insect, the enzyme was detected lining the inside of the insect cuticle (4.7b).

4.1.3.4 Electron Microscopy

Aphids for light microscopy were sectioned further to a thickness of 100nm for electron microscopy. Immunogold labelling was performed on control and Vertalec infected insects using antibody raised to Vertalec PR1-like enzyme at a concentration of 1:100. No immunogold labelling was observed on sections of non-infected insect or infected insects directly applied with gold labelled secondary antibody without primary antibody.

At an early stage of infection (2d - plate 4.8) the fungus had already crossed the cuticle and invaded the tissue underneath. A blastospore or transverse section through a hyphal fragment can be clearly seen within the insect. Gold labelling is apparent within the cuticle itself (around a possible pore canal or some sort of disturbance created by the fungus?) At this stage of mycosis, the enzyme is not present within the internal insect tissues.

As infection develops, elements of the fungus can be seen throughout the insect (plate 4.9) - here apparently within the fat body cells. An elongated fungal structure, a hyphal body, is shown and a spherical structure, which could be a blastospore or a

hyphal fragment sectioned transversely. In both instances, immunogold labelling is found inside the fungus and the fungal cell wall. The degree of immunostaining, albeit low in most cases, is variable with each fungal body (plate 4.10) but is always found directly on or in the fungal wall. Very little or no labelling can be seen outside of the fungus.

Plate 4.8 Early infection by Vertalec. 100nm sections of aphid 2d post application of conidia were taken and incubated with antibody at 1:100 and developed using a secondary antibody-gold conjugate. Sections were stained with uranyl acetate and lead citrate for visualisation by electron microscopy. Areas of labelling are highlighted (>).

Bar represents 1μm

CU = Cuticle

BL = Blastospore or hyphal fragment sectioned transversely

PC = Pore canal?

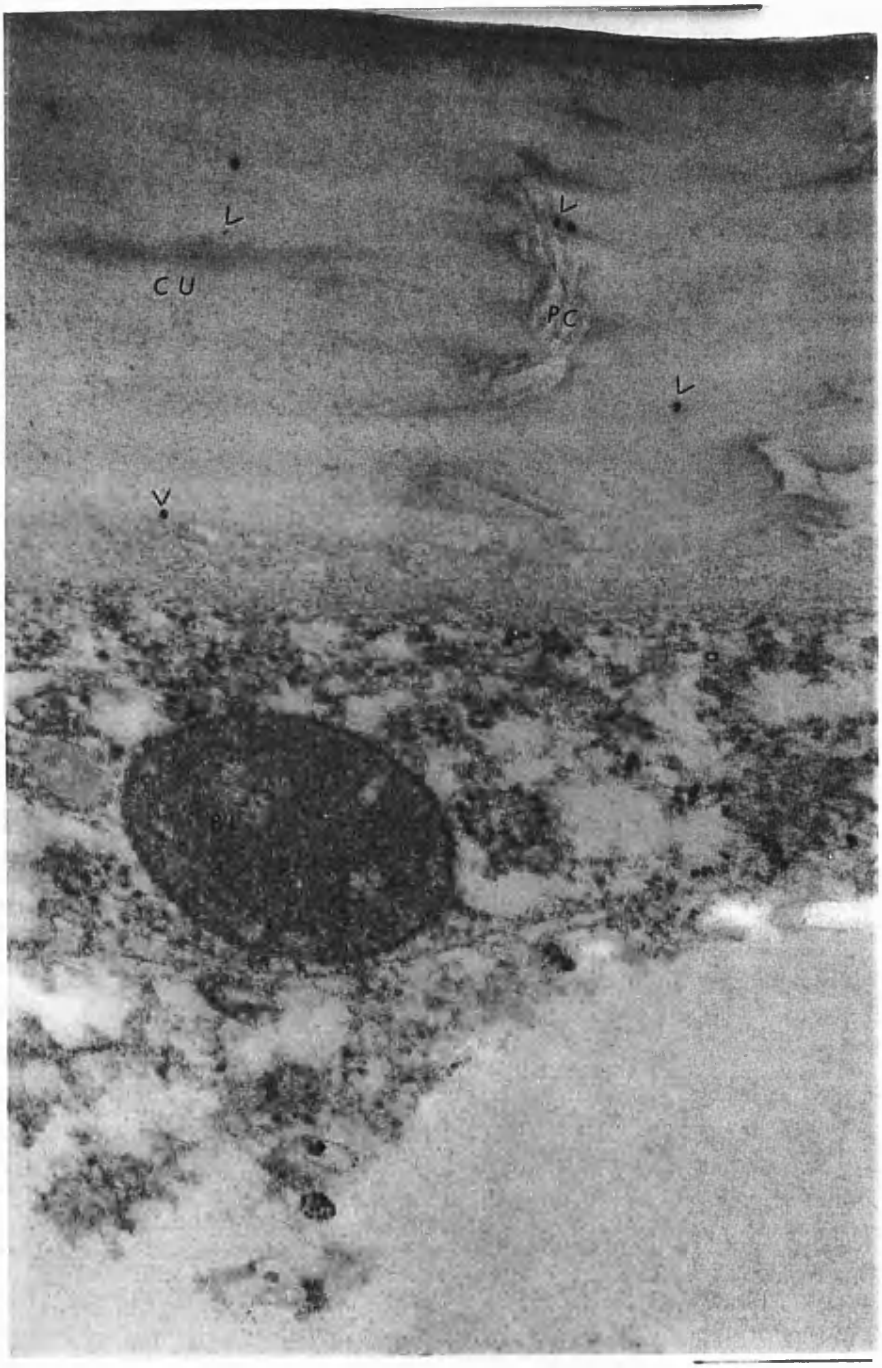

L
L
CU
PC
L
V

Plate 4.9 Mid to late infection of aphids by Vertalec. 100nm sections of aphid 3-4 days post application of conidia were taken and incubated with antibody at 1:100 and developed using a secondary antibody-gold conjugate. Sections were stained with uranyl acetate and lead citrate for visualisation by electron microscopy. Areas of labelling are highlighted (>).

Bar represents 1µm

H = Hyphal fragment

BL = Blastospore or hyphal fragment sectioned transversely

F = Lipid Droplet

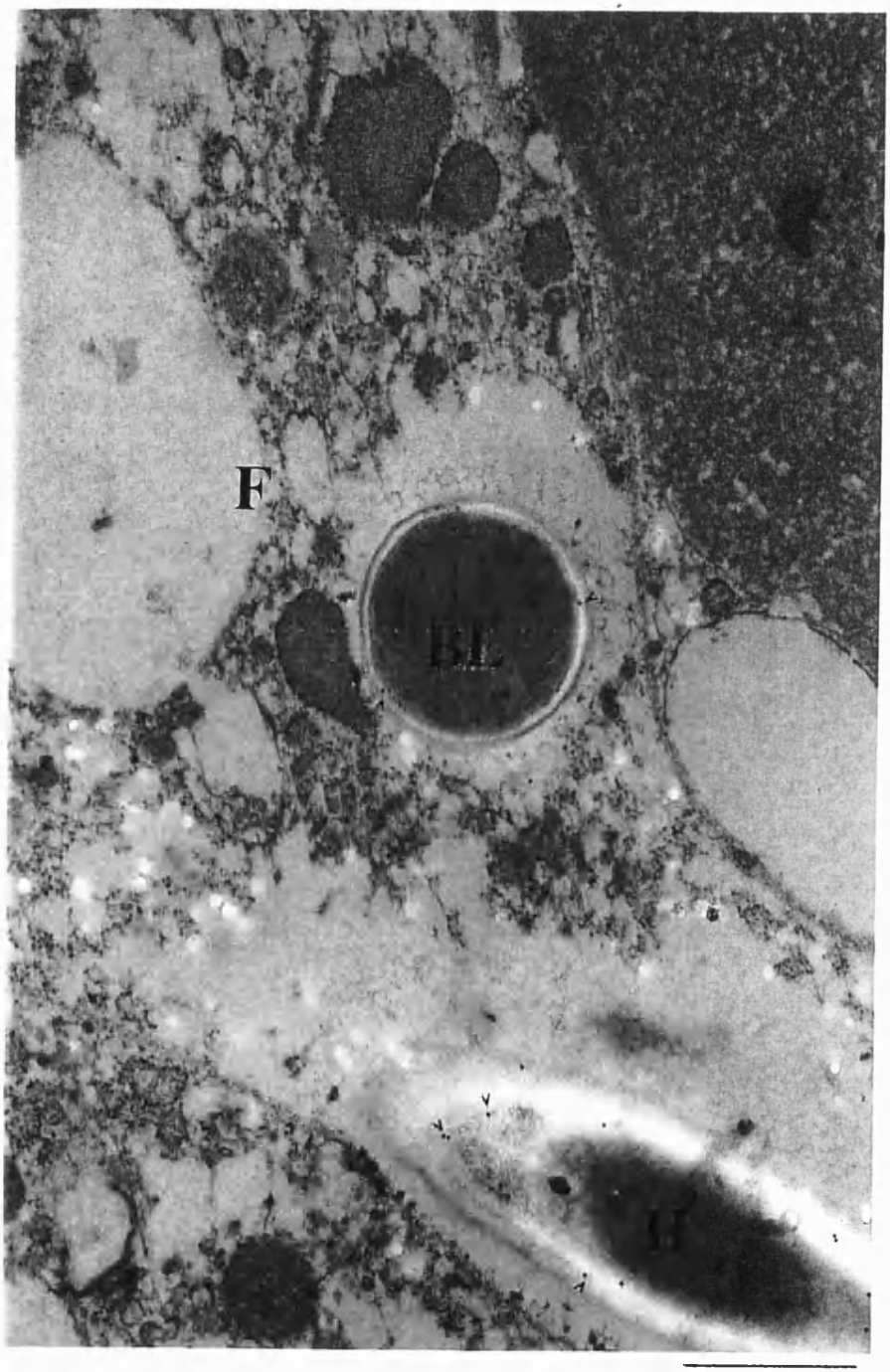

F
BL

Plate 4.10 Mid to late infection of aphids by Vertalec. 100nm sections of aphid 3-4 days post application of conidia were taken and incubated with antibody at 1:100 and developed using a secondary antibody-gold conjugate. Sections were stained with uranyl acetate and lead citrate for visualisation by electron microscopy. A hyphal fragment (a) and blastospore (b) is shown. Areas of labelling are highlighted (>)

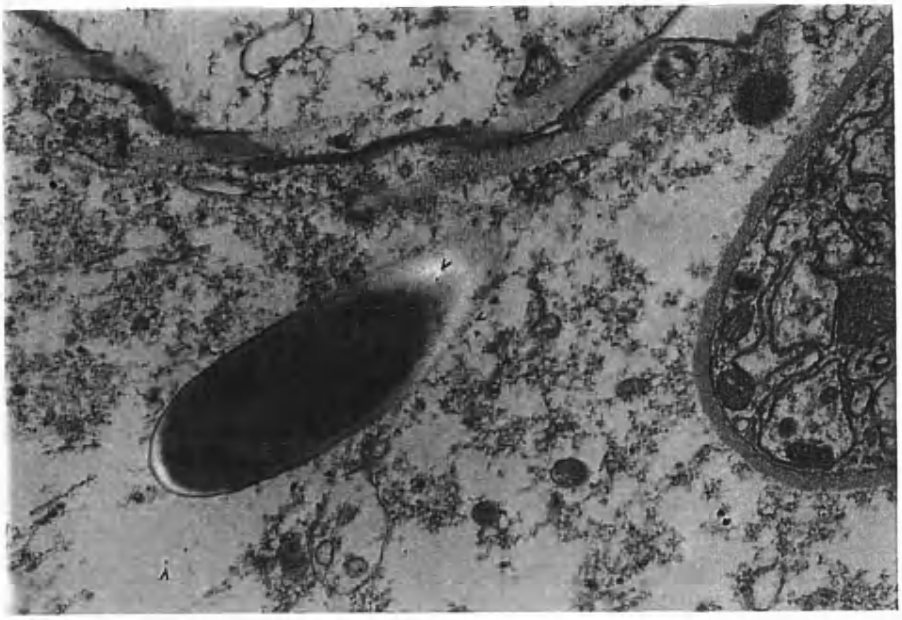
Bar represents 1µm

H = hyphal fragment

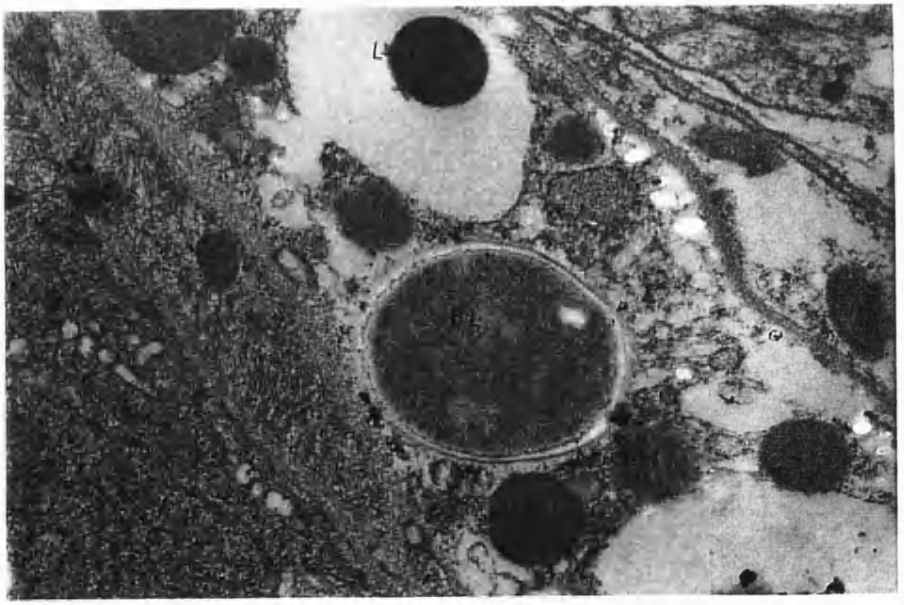
BL = blastospore or hyphal fragment sectioned transversely

L = lipid droplet

MT = mitochondrion



a



b.

4.2 Discussion

Enzyme assays of homogenates of control aphids revealed low activity against the PR1-substrate N-Suc-Ala-Ala-Pro-Phe-AMC. Serine proteases have been found in insect haemolymph where they regulate the prophenoloxidase activation and coagulation cascades (Gillespie and Kanost 1997). Similar enzymes have been found in insect cuticle and moulting fluid where they effect recycling of cuticular protein at ecdysis (Samuels et al. 1993). Digestive enzymes in the alimentary canal are an additional source of serine proteases (Christeller et al. 1992, Rymerson and Bodnaryk 1995).

Thus, comparisons between Vertalec and KV42 infected insects must take into account low levels of endogenous protease. Within Vertalec infected insects, although PR1-like activity on day 2 was greater than that of the controls, it was then repressed until death of the host and emergence of the fungus. This may be interpreted as production during penetration of the cuticle but absence during proliferation within the haemolymph. Indeed, St Leger et al. (1996b) and Gillespie (1995) did not detect PR1 produced by wildtype isolates of *M.anisopliae* in haemolymph until very late in infection. By contrast, high levels of PR1-like activity compared to that of controls, were detectable throughout infection (day 1 - day 6) for KV42 infected insects. Both isolates appeared to kill the host at approximately day 4 suggesting that differences in enzyme titre did not cause variation in rates of kill.

Isolate KV42 of *V.lecanii* produced PR1-like isoforms that were not sensitive to carbon or nitrogen repression (chapter 3). Vertalec showed a level of repression by

nitrogen and total repression of PR1-like activity by carbon. Catabolite repression may be an important factor regulating the production of PR1, not just on the surface of the host (St Leger et al. 1989) but also once the fungus begins to colonise the haemocoel. An isolate not subject to catabolite repression, such as KV42, may produce the enzyme within the nutrient rich haemolymph even very early during infection.

Apart from the effects of catabolite repression, delayed production of PR1 in the insect haemolymph during infection has been attributed to sensitivity to enzyme inhibitors (Boucias and Pendland 1987). Unlike *M.anisopliae* mycosis in *M.sexta*, where dark melanic patches were observed in the cuticle (St Leger et al. 1988d, 1996c), no darkening of aphid cuticle was observed prior to emergence of *V.lecanii* in this study. Furthermore, pure PR1 from *M.anisopliae* caused darkening of *M.sexta* haemolymph *in vitro*, whereas similar enzymes from *V.lecanii* had a very slow/negligible effect on haemolymph from this insect. Unlike the chymoelastases from *M.anisopliae* those from *V.lecanii* do not activate the phenoloxidase cascade.

A very basic chymoelastase-like enzyme was detected in control and infected aphids over the course of the experiment using IEF, gelatin overlay and enzyme assay. However, these methods were not sensitive enough for quantitative assessment. Thus it is not possible to establish whether this isoform contributed to elevated chymoelastase during infection.

The appearance of isoforms of protease in infected aphids which are absent from control aphids is consistent with a fungal origin. For Vertalec infected insects, an isoform of PR1-like enzyme at pI 8.0 was detected on day 4 of infection - as insects

began to die. This form of the enzyme was not observed in liquid culture on aphid or locust cuticle, suggesting that it is specifically induced in the living host. Similarly, some plant pathogens such as *Rhizoctonia solani*, and *Botrytis* spp. produce different isoforms of pectic enzymes within the plant to *in vitro*, this may be attributable to pH conditions within the host (reviewed by Cooper 1976). As the fungus began to emerge from the aphid, PR1-like activity, in addition to that observed at pI $\geq$9.5, could be detected at the sample loading position (6.85-7.35). It is possible that a new form of the enzyme of less basic pI is produced. However, fungal growth in and on the insect was so great at this point in infection, that fungal protein constituted a large proportion of the total protein applied to the gel. Thus it is highly probable that much of this protein remained in the area of sample loading and did not enter the gel. Similar findings on emergence of the fungus were observed for KV42 infected aphid homogenates.

On days 3 and 4 of infection, KV42 produced inside the insect two isoforms of PR1-like enzyme at pI 8.0 and 8.19, these forms of the enzyme were not found within control insects but were observed on cuticle in culture. The production of these isoforms may account for the increased PR1-like activity detected within the host. Other forms of chymoelastase were not detected using this method.

On day 4, in Vertalec infected aphids, a protease of pI 4.2 was also detected, this did not show any activity towards the PR1 or PR2 substrates. This enzyme was detected also on aphid cuticle but not locust cuticle (chapter 3). Perhaps this enzyme is induced by a host specific peptide sequence. St Leger et al. (1998) reported that *M.anisopliae* (ARSEF strain 2575) produced a number of low pI aspartyl proteases on insect cuticle

only under acidic conditions. It is possible that this new protease only found on infection of aphid cuticle and within the aphid host, is an aspartyl protease that may be induced by a low favourable pH found within the aphid cuticle.

Activity assays are relatively insensitive and cannot provide conclusive evidence of the origin of protease production within the host. The use of polyclonal antibodies for ultrastructural localisation is potentially a much more sensitive and specific technique. For example, no chitinase activity was detected during early stages of penetration of *Manduca sexta* cuticle by *M.anisopliae* (St Leger et al. 1987b), however, immunocytochemistry experiments showed that chitinases were present, albeit at low levels (St Leger et al. 1996a).

The basic (pI >9.5) PR1-like enzyme from Vertalec cuticle cultures was successfully purified to a single band on SDS-PAGE, as visualised by Coomassie blue and silver stain. The analogous PR1-like enzyme of KV42 was not purified to a single band, but for the purposes of raising antibodies, it could be excised cleanly away from the other contaminating bands. The PR1-like enzymes from Vertalec and KV42 were estimated to be 30.5kDa and 31.8kDa respectively as determined by SDS-PAGE and Coomassie Staining. Isoforms of PR1 from *M.anisopliae* (ARSEF 2575, formally ME1), of pI 10.2, 9.8, 9.3, 9.0 were characterised as being 30.2kDa, 28.5kDa, 29.5kDa and 31.5kDa in size, respectively (St Leger et al. 1994). Thus the purified PR1-like enzymes of *V.lecanii* are similar in size to those of *M.anisopliae*

Polyclonal antibodies were raised successfully to PR1-like enzymes from both isolates of *V.lecanii* in all four rabbits. Interestingly, PRE-bleed serum from each rabbit

showed a level of recognition of crude cuticle culture filtrates and purified enzyme from each isolate, even though the rabbits had not previously encountered the fungus. However, Western Blot analysis, (results not shown) indicated a smear down the blot for crude filtrate, indicating a non-specific recognition of fungal proteins. The reasons for this non-specific antibody recognition are not understood but are not uncommon (Dave Hornsey, University of Bath, personal communication).

After the first injection, the serum of each rabbit only bound weakly to the antigen, however the strength of this reaction increased with subsequent boosts. Western blot analysis showed that serum from Vertalec and KV42 boosted rabbits recognised a single band at a size corresponding to those of PR1-like enzymes from crude cuticle cultures of Vertalec and KV42 respectively. Repeat boosts with antigen resulted in polyclonal antibodies specific for PR1-like enzyme. Peak titre was obtained after four boosts for Vertalec and five for KV42. Using a secondary antibody bound to horse-radish peroxidase system, the limit of recognition of crude filtrate and pure enzyme was at 1:1000 dilution of antibody, although a much clearer and significant response was observed at 1:100 -1:500. Blots of iso-electric focusing gels were not as sensitive, as blots of SDS-PAGE gels, even using the Avidin/Biotin system, although it would appear that antibodies raised to each isolate could recognise proteins corresponding to iso-electric points of other PR1-like enzymes in addition to the pI $\geq$9.5 isoform. Thus antibodies may not be isoform specific at the dilutions tested. Future studies raising antibodies to each isoform of protease from these isolates would be needed to understand the serological relatedness of the isozymes. Variation in response to antibody may reflect differences in amino acid sequence (Gillespie et al. 1998), which in turn could affect function. Strategic amino acid substitutions can affect cuticle

binding and thus activity of PR1 from *M.anisopliae* (St Leger et al. 1986, 1992). Given that the protein composition of insect cuticle varies between insect species, variations in PR1 sequence could influence host range. Cuticle proteins will also vary in charge, thus cuticle degradation may vary on different regions of the insect body (Gillespie et al. 1998) and between insect species (Bidochka and Khachatourians 1994a). Antibodies raised to an isoform exclusive to isolate ME1 of *M.anisopliae*, failed to cross react with PR1 from isolate V245 of *M.anisopliae* or VCP1 from *V.chlamydosporium* (Segers et al. 1995). The authors suggest that this could be due to absence of epitopes on this particular isoform and that these differences could account for variation in host specificity between isolates of *M.anisopliae* by means of subtle differences in substrate specificity.

Antibodies raised to PR1 from Vertalec recognised analogous enzymes from isolate KV42 and *M.anisopliae* at 1:1000 dilution using the Avidin/Biotin system, although the reaction was weaker than that of the homologous enzyme at the same concentration. The antibody showed little or no binding to PR2 at this concentration, suggesting that the antibody was specific for epitopes on a chymoelastase enzyme. Dilution increased the specificity of the antibody, at 1:4000 only a strong signal was obtained for pure Vertalec enzyme. Antibodies raised to PR1-like enzyme from KV42 bound equally well to enzyme from either isolate of *V.lecanii* at 1:1000, but were weak against proteins from *M.anisopliae*. Only a very weak signal was observed to either *V.lecanii* isolate at 1:4000. These polyclonal antibodies, although recognising similar epitopes between *V.lecanii* isolates, may not recognise similar epitopes from *M.anisopliae*. The dilution of antibody used and technique employed are likely to determine the extent of cross reactivity. Shimizu et al. (1993), for example, found that

in Ouchterlony diffusion tests that antibodies raised to a protease from an isolate of *B.bassiana* cross reacted with all other strains of this species tested, and two isolates of *B.brongniartii*, however they did not cross react to proteases of isolates of *M.anisopliae* and *Paecilomyces fumosoroseus*, suggesting an immunological distinction. However, by using the much more sensitive technique of sandwich Enzyme Linked Immunosorbent Assay, a level of cross reactivity could be observed to all isolates, suggesting that all proteases shared common antigens; presumably at the active site where there is a degree of sequence homology between serine proteases.

In the present work antibodies (at 1:1000 dilution) raised to Vertalec enzyme cross react with crude filtrate from KV42, *M.anisopliae*, *B.bassiana*, and an aggressive strain of *Trichoderma harzianum* (a pathogen of the common mushroom). At a much higher dilution, the antibodies were more specific, predominantly only binding to filtrate from Vertalec. These polyclonal antibodies did not, however, bind well to another isolate of *V.lecanii*, the whitefly strain, Mycotal. Thus antibodies for PR1-like enzymes may not only be species specific, but also isolate specific - again possibly reflecting slight structural differences in the enzyme that could affect its ability to bind to and degrade substrates and perhaps, the potential host range of the isolate (Gillespie et al. 1998, Segers et al. 1995. Antibodies raised to KV42 did not cross react well with any of the culture filtrates other than its own or that of Vertalec; in fact the stronger signal to the latter may suggest that the Vertalec protein is more antigenic.

The polyclonal antibodies were used successfully to immunostain mycosed aphids; no staining was observed within control aphids. Given the low dilution of antisera used

(1:2000) it is reasonable to conclude that the response was due to fungal protease. Furthermore the pattern of staining with time was consistent with changes in chymoelastase activity detected in aphids infected with the 2 isolates of *V.lecanii*.

Over the first two days post infection, very little immunostaining was observed within Vertalec infected insects, except at the posterior end. Commonly, the rear, where honey dew is produced, of living but *V.lecanii* infected aphids, is covered with a visible growth of the fungus. Presumably, this high nutrient environment supports extensive surface fungal growth. However, once the carbon rich source is depleted, the isolate may proceed to invade the insect, producing cuticle-degrading enzymes along the way. Schreiter et al. (1994) found that *V.lecanii* typically colonised the surface of the western flower thrip, *Frankliniella occidentalis* prior to or simultaneously with penetration of the host cuticle.

Early during mycosis, immunostaining of Vertalec infected aphids was restricted to the vicinity of posterior abdominal cuticle. This is consistent with the low enzyme activity in homogenates of infected aphids until very late in infection. The production of chymoelastase within the host appears to coincide with an increase in fungal growth and degradation of the surrounding tissues. There are a number of possible triggers for this derepression of the enzyme. Firstly, protease inhibitors or other host defences may influence the activity of fungal enzymes in host haemolymph (Boucias and Pendland, 1987). Secondly, a number of cuticle degrading enzymes such as proteases, chitinases and NAG-ases produced by entomopathogens

are subject to catabolite repression (reviewed in Bidochka et al. 1997). PR1-like enzymes of Vertalec are particularly sensitive to carbon. Given the carbon rich environment inside these sap-feeding aphids, it is possible that carbon repression is a key factor in controlling chymoelastase production. The requirement for cuticle degrading enzyme production, once accessible nutrients have been depleted, may in itself be a trigger for the fungus to leave the host. Very late in mycosis, immunostaining of PR1-like protease in Vertalec infected aphids was widespread on the inside of the cuticle. This may denote cuticle-induced synthesis as a prelude to re-emergence of the fungus from the cadaver.

Environmental factors such as pH are also likely to have a significant effect *in vivo*. St Leger et al. (1998) found that even in the presence of an inducing substrate, an isolate of *M.anisopliae* did not produce PR1 under acid conditions. The pH within an aphid is not known, however if it is acid that may preclude the production of PR1, in which case this enzyme is likely to remain repressed until the action of other enzymes, such as the PR2-like trypsins, elevate the pH (St Leger et al. 1996a).

The strategy of enzyme production by isolate KV42 as indicated by *in vitro* and *in vivo* experiments was dramatically different to that of Vertalec. Even early during infection, immunostaining of the enzyme occurred extensively in the tissues immediately beneath the cuticle (particularly fat body). As infection proceeded, enzyme production within host tissues was enhanced, but there were no signs of wide-spread tissue degradation as observed with Vertalec on day 4. Production of PR1-like enzymes by KV42 must somehow circumvent the factors repressing Vertalec protease production. The remarkable insensitivity to accessible sources of carbon or nitrogen shown by KV42 *in*

vitro compared to Vertalec is likely to be very important in determining the isolates' strategies. A further difference between isolates was the lack of fungal growth within KV42 infected insects during early stages of infection, compared with obvious hyphal fragments and blastospores within Vertalec infected insects. Similarly, very little fungal material was observed within thrips at the time of death when infected with an appropriate isolate of *V.lecanii* (Schreiter et al. 1994). It is possible that excessive enzymic degradation is sufficient, in itself, to kill the insect without extensive fungal colonisation of the haemocoel. Future studies probing infected aphids with antibodies raised to fungal cell walls could clarify the findings here. Furthermore, incorporating a positive control - injecting pure PR1 into control and infected insects would help confirm that the antibodies are binding to fungal and not insect proteases.

The developing offspring were not affected by the fungus and no PR1 could be detected in or around them; the ovariole membrane appears to be resistant to the fungus. This explains why living juvenile aphids sometimes emerge from heavily sporulating, apparently dead mothers (Roditakis, personal communication). It seems likely that these aphids will acquire a lethal dose of conidia during birth. However, feeding prior to death will prolong damage to the crop after application of a mycoinsecticide based on *V.lecanii* and delay economic control of the aphid population.

PR1-like protease was ultrastructurally located to aphid cuticle during fungal penetration. Small number of gold particles were seen in the cuticle of Vertalec infected aphids but were absent from the epidermis and fat body beneath, though

experiments on cuticle excised from the host, thus the pattern of production once inside the insect was not examined. Late in infection, once the mycosis has become established, PR1 production is closely associated with spores/ hyphal fragments of Vertalec, throughout the insect tissue, it also appears to correlate with areas of degradation of the host. As found in early stages of penetration for *M.anisopliae* (Goettal et al. 1989), the enzyme itself is clearly bound to and within the hyphal wall, however here within the haemolymph, very little is observed around the fungus - presumably no enzyme is released until required, i.e. very late during infection when the fungus exits the insect.

In conclusion, sensitivity of PR1-like enzymes to catabolite repression found *in vitro*, may dictate production *in vivo*. An isolate of *V.lecanii* whose proteases were repressed by low molecular weight carbon and nitrogen, Vertalec, did not produce PR1-like enzymes during early stages of mycosis, the enzyme was predominantly bound to the fungal wall. After some growth within the insect, and just prior to/ concomitant with death of the host, PR1 was released and large sections of tissue were degraded. In contrast, KV42, an isolate not repressed by carbon or nitrogen, produced PR1-like enzymes within the insect very early during mycosis. Furthermore, no fungal growth was seen, and tissues remained largely intact until after death of the host.

5 General Discussion

In order to fully realise the potential of entomopathogens in the pesticide market, they must continually undergo improvements to enable them to compete with their chemical counterparts. There are a number of ways that researchers can try and improve mycopesticide performance in addition to adapting formulations and application methods, Genetically engineering existing fungal isolates by direct addition or upregulation of one or more genes coding for pathogenicity/virulence determinants (St Leger and Roberts 1997); by parasexual crossing or protoplast fusion of promising isolates (Heale et al. 1989) or by application of mixtures of isolates to the target pest.

Whatever method is employed, an understanding of the disease cycle is essential. For genetic engineering, information is needed on the genes that control pathogenesis and/or specificity. Furthermore, in order to select mixtures or to cross isolates for improved control, the desirable traits in disease development must be identified. Individual isolates of a fungus may not significantly differ in their effective rate or efficiency of kill, but may employ different disease strategies to achieve successful infection. 2 isolates of *M.anisopliae* ME1 and 703, for example, kill the host primarily by toxins and extensive growth respectively. A combination of both the capacity for rapid growth as well as toxin production in one mycopesticide by genetic engineering, parasexual crossing or by combining two isolates into a mixture could improve the speed and efficiency of pest control.

Given its reputed importance in pathogenicity, PR1 has already been the target for genetic manipulation of *M.anisopliae* in an attempt to improve virulence of the mycoinsecticide (St Leger et al. 1996c). The ability to produce PR1-like enzymes on insect cuticle, and under conditions that might otherwise be repressive may also be an important consideration for isolate improvement programmes for *V.lecanii*. All isolates produced PR1-like activity on locust cuticle as the sole source of carbon and nitrogen, however differences were observed between isolates in the levels of activity and the timescale of production in culture. If PR1 production by *V.lecanii* affects the extent and rate of penetration and mycosis development in the host, and differences between isolates seen in culture are also observed *in vivo*, then perhaps a combination of a high PR1 producer and an early producer within a mixture could give promising results. Production *per se* does not reveal a great deal about individual isolates, it is the ability of the fungus to regulate enzyme production in response to host specific cues that is likely to affect the different strategies of mycosis and host specificity.

Insect cuticle, as the sole source of carbon and nitrogen, significantly induced chymoelastase activity above that of basal salts controls for all isolates of *V.lecanii* tested. For each isolate tested, there was no significant difference in activity between host (aphid) or non-host (locust) cuticle, and the same pattern of isoforms was produced on either cuticle. Presumably the factors needed to induce activity are present within both cuticles, and thus overall induction of PR1-like enzymes does not dictate host specificity. A comprehensive study, testing cuticles from a wide range of insect groups and entomopathogenic fungi isolates from different hosts is needed to consolidate such a statement.

Contrasting strategies of induction were observed, however, for aphid and whitefly derived isolates of *V.lecanii*. All aphid isolates showed some level of induction by the chitin component of the cuticle, apparently attributable to the release of the monomeric constituent of chitin, N-acetylglucosamine. The whitefly isolate, Mycotal, was not induced by GlcNAg, but was induced non-specifically by a proteinaceous component of the cuticle. Each isolate produced its own distinct array of protease isoforms, which themselves were induced by different substrates. All isoforms produced by Mycotal on insect cuticle were also induced non specifically by protein. In contrast, the aphid isolate, Vertalec, produced one isoform specifically induced by cuticle and no other substrate. Furthermore, only one isoform was induced by N-acetyl glucosamine. It is possible that for Vertalec, each isoform has a role during penetration - those that are induced by a proteinaceous component of the cuticle are released first, they then open up the chitin fibrils of the cuticle for hydrolysis by chitinases which releases GlcNAg which regulates the control of the other isoform through an inducer-repressor mechanism. The PR1-like enzymes of Mycotal are presumably only produced on contact with protein, and are not regulated in the same way as Vertalec. Induction alone may not dictate the contribution of PR1 to determining host specificity, the ability of the proteases to breakdown the cuticle and release nutrients for the fungus must be important. In this study, many of the isoforms produced by aphid derived isolates were more efficient at breaking down the aphid cuticle than locust cuticle, whereas no clear differences were observed for Mycotal, the whitefly strain. The charge of the proteins in the cuticle, and the charge of the isoforms may dictate the ability of the protease to bind and thus digest the cuticle. In addition to electrostatic adsorption, location of the protein in the protein/chitin matrix, protein-chitin interactions and protein glycosylation may also dictate susceptibility to attack

(Bidochka and Khachatourians 1994a,b). A comparative study of the composition of aphid and whitefly cuticle, may enable some understanding of the strategies of PR1 production by these two isolates.

It would be naive to assume that PR1-like enzymes alone affect the specificity of entomopathogenic fungi, the fungus produces a number of other extracellular enzymes such as trypsins, aminopeptidases, dipeptidylpeptidases, aspartyl proteases, chitinases and lipases that all work together in enabling the fungus to enter into the host (St Leger et al. 1986b, 1987a, 1993a & 1998). In this study two acid proteases were, for example, only observed on aphid cuticle and not locust cuticle. Furthermore, triggers for adhesion and germination are also likely to be important in determining the host range of the fungal isolate. However, for the purposes of mycoinsecticide improvement, it would be interesting to see if combination of Mycotal and Vertalec within one mixture could actually widen the host range and improve virulence - by more effectively degrading the target host cuticle.

Sensitivity to catabolite repression is also likely to be a key factor controlling protease production *in vivo*, irrespective of an inducing factor. In isolates of *V.lecanii* and *M.anisopliae* (Paterson et al. 1994a) PR1 production is switched off under nutrient rich conditions. This is an evolutionary adaptation to an opportunistic pathogen's way of life, in that it allows conservation of resources. If a fungus can already gain enough nutrients to sustain growth, there is no necessity to initiate a pathogenic relationship with a host insect. The honey dew of sap-feeding aphids, commonly found on the surface of the insect itself, predominantly contains carbon, in the form of readily available sugars from the host plant. Sugars are also found in high concentrations in

the haemolymph. Thus an isolate capable of producing PR1-like enzymes even in the presence of soluble carbon is not only likely to produce such enzymes earlier on the surface of the host contributing to an earlier penetration, but also early during invasion of the haemocoel. Such enzymes could then contribute directly to death of the host. Free amino acids also prevail within insect haemolymph, however this study suggest that their concentration *in vivo* is not inhibitory to these PR1-like enzymes. The potential repressive effect of amino acids on fungal proteases is worthy of future study.

Chymoelastase production by 4 of the 5 isolates of *V.lecanii* tested here, although apparently controlled by multiple regulatory circuits was fundamentally repressed by the presence of available carbon. All PR1 isoforms from Vertalec were repressed by low molecular weight carbon in cuticle cultures. In contrast, all isoforms of PR1-like enzyme from one isolate, KV42, were insensitive to the presence of carbon. Isolates of *V.lecanii* are commonly seen to grow extensively on the surface of the insect prior to penetration - because of the readily available nutrients (Schreiter et al. 1994). If KV42 still produces PR1-like enzymes even in the presence of honey dew, the fungus may enter the host before extensive growth. Initiation of infection as soon as possible after application of conidia, is highly desirable for pest control programmes. Low molecular weight carbon was neither repressive for PR1 production by KV42 in cuticle nor in BSA containing cultures. This has implications for protease production within the very different nutrient rich environment of the host haemolymph. Interestingly, *in vitro* differences in protease regulation between Vertalec and KV42 were also seen *in vivo*.

PR1-like activity in Vertalec infected insects was largely repressed until the death of the host, but that of KV42 infected insects was high throughout infection. Both these isolates exhibited a similar rate of kill of the aphid host, suggesting that suppression of PR1-like activity does not affect actual virulence of pathogens but may dictate the strategy of invasion employed. Here for example, Vertalec was seen to proliferate within the host without prior enzyme production until very late in infection - however, at this stage, large sections of tissue were degraded. Yet for KV42, fungal development within the host was minimal, enzyme production was high and tissue degradation was not as apparent as for Vertalec. It is possible that Vertalec kills the host predominantly by proliferation and only produces degradative enzymes when available host nutrients are depleted, furthermore, these enzymes are required for exit of the fungus through the cuticle, to enable it to re-infect other aphids. For KV42, PR1-like enzymes may be a means of deriving nutrition from the host throughout infection, furthermore, the fungus may actually kill the host using these enzymes rather than extensive growth. Similarly, Graystone (unpubl.) found that KV42 was one of the first isolates to be detected within the insect following inoculation yet showed negligible proliferation in the host until death and re-emergence from the cadaver. Vertalec was not the first to enter the host, but grew profusely once the cuticular barrier had been crossed. Although there may be a number of factors controlling protease production within the host, such as environmental factors and host defences, these findings suggest that the sensitivity of PR1 to catabolites may be important in determining the role that the enzyme plays throughout infection, not just in penetration.

One isoform of protease was produced by Vertalec only within the aphid host itself, and not in cuticle cultures. The specific induction of a protease *in vivo* needs further attention. The study of regulation of PR1-like enzymes by isolates of *V.lecanii* should be widened to observed developmental regulation - to relate to the changes in growth within the insect, and pH regulation - pH changes throughout the cuticle and within the haemolymph and host tissues could be very important in affecting PR1 production.

Preliminary studies at Bath using Vertalec and KV42 in mixtures have proved largely unsuccessful. Application of the two isolates does not result in a synergistic reduction in time to death, but in fact, time to death appears to be dictated by Vertalec (Roditakis unpubl). This would suggest that the two isolates compete with one another. Similarly Chandler et al. (1993) observed competitive interaction between strains of *V.lecanii* on whitefly and aphid hosts. It is possible that the sequential application of the 2 isolates may prove more effective. That is, by applying isolate KV42 initially, allowing it to penetrate the host very quickly to begin producing toxic enzymes within the insect and thus predispose the aphid to further infection by the later applied Vertalec, which will proliferate much faster within the host.

Studies on the regulation of PR1-like enzymes of isolates of *V.lecanii* have highlighted differences in production, mode of induction and sensitivity to catabolite repression between isolates and isoforms. Whether regulation is such that it allows the enzyme to contribute to death of the host or just be tool for acquiring nutrients, utilisation of mixtures could still be an important way of maximising the role of PR1 in the infection process.

If mixtures prove unsuccessful, it may be possible to cross parasexually the required isolates, or genetically engineer existing isolates. It is not yet known whether or not these isoforms of protease from *V.lecanii* are the product of one gene or a number of genes. Identification of the genes encoding proteases specifically induced by the cuticle may be important for mycopesticide improvement programmes. St Leger et al. (1996c) have already shown that multiple copies of a gene encoding for PR1 in *M.anisopliae* proved to be highly toxic and led to an increased virulence against the target pest. Selecting for genes whose products are constitutively produced and not repressed by carbon would be highly desirable for an even more potent mycoinsecticide.

For any study, the close examination of just one factor, i.e. PR1-like production, is limited in what it can reveal about pathogenesis. It is essential that future work investigates the whole cuticle-degrading enzyme complex incorporating not just chymoelastases but trypsins, aminopeptidases, dipeptidylpeptidases, aspartyl proteases, chitinases and lipases. This will give a clearer understanding of the different roles of extracellular enzymes for different isolates of a fungus in pathogenesis.

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Appendices

APPENDIX 1 - ADDITIONAL REAGENTS/SOLUTIONS

1.1 NELSON-SOMOYGI REDUCING SUGAR ASSAY

Reagent A:

Na_2CO_3	25g
Sodium potassium tartrate	25g
$NaHCO_3$	20g
Na_2SO_4	200g
Distilled water	800ml

Make up to 1 litre with additional distilled water.

Reagent B:

$CuSO_4.5H_20$	15%

1 or 2 drops of conc.H_2SO_4 per 100ml

Reagents A and B are mixed in the ratio 25:1 and allowed to stand for 30 min prior to use.

Reagent C:

Made up in clean, acid washed glassware.

Ammonium molybdate	25g
Distilled water	450ml

Once dissolved add 21ml H_2SO_4.

3g Na_2HAsO_4 (arsenic) is dissolved in 25ml distilled water, added to the ammonium molybdate solution and incubated at 37°C for 24-48h in the dark.

1.2 POLYACRYLAMIDE GEL ELECTROPHORESIS

Resolving Gel (12.5%)

30% Acrylamide Solution (Protogel)	4.1ml
1.5M Tris-HCl (pH 8.8)	2.5ml
MQ water	3.33ml
10% APS (ammonium persulphate)	100µl
10% SDS (sodium dodecyl sulphate)	75µl
TEMED	10µl

Stacking Gel

30% Acrylamide solution	750µl
0.5M Tris-HCl (pH 6.8)	1250µl
MQ water	3000µl
10% APS	50µl
10% SDS	50µl
TEMED	5µl

Protein Markers (BioRad)

200 kDa	Myosin
116.25 kDa	B-galactosidase
97.4 kDa	Phosphorylase
66.2 kDa	Serum albumin
45 kDa	Ovalbumin
31 kDa	Carbonic anhydrase
21.5kDa	Trypsin inhibitor
14.4 kDa	Lysozyme
6.5 kDa	Aprotinin

Reservoir buffer stock (10x)

Tris base	30.3g
Glycine	144g
SDS	10g

Make up to 1 litre with distilled water.

SDS Sample Equilibration Buffer

0.5M Tris/HCl pH 6.8	12.5ml
10% SDS	23ml
B-mercaptoethanol	5ml
Glycerol	8ml
Bromophenol Blue 0.05%	2.5ml
49ml distilled water	49ml

APPENDIX 2 - Statistical Analyses of Regulation Experiments

Oneway Analysis of Variance was performed on each data set, testing the hypothesis that the enzyme activities were from the same source.

Fishers pairwise comparisons highlighted where treatments were significantly different from each other. (*Sig. shows were the treatments are shown to have a significant effect using intervals for (column level mean) - (row level mean), critical value = 1.992)

2.1 INDUCTION

1a) Mycotal 16 hours

F=192.22 P= <0.000 (4, 75 df)

	Cuticle	Chitin	KOH Chitin	BSA	Control
Cuticle					
Chitin	*Sig				
KOH Chitin	*Sig	*Sig			
BSA	*Sig	*Sig	*Sig		
Control	*Sig	*Sig	*Sig	*Sig	

1b) Mycotal 36 hours

F=247.29 P= <0.000 (4, 75 d.f)

	Cuticle	Chitin	KOH Chitin	BSA	Control
Cuticle					
Chitin	*Sig				
KOH Chitin	*Sig	*Sig			
BSA	*Sig	*Sig	*Sig		
Control	*Sig	*Sig	*Sig	*Sig	

2a) KV54 16 hours

F=97.64 P= <0.000 (4, 75 d.f)

	Cuticle	Chitin	KOH Chitin	BSA	Control
Cuticle					
Chitin	*Sig				
KOH Chitin	*Sig	*Sig			
BSA	*Sig	*Sig	*Sig		
Control	*Sig	*Sig	*Sig	Not Sig.	

2b) KV54 36 hours

F=11.49 P= <0.000 (4, 75 d.f.)

	Cuticle	Chitin	KOH Chitin	BSA	Control
Cuticle					
Chitin	*Sig				
KOH Chitin	Not sig.	*Sig			
BSA	*Sig	*Sig	Not sig.		
Control	*Sig	Not sig.	*Sig	*Sig	

3a) Vertalec 16 hours

F=152.41 P= <0.000 (4, 75 d.f)

	Cuticle	Chitin	KOH Chitin	BSA	Control
Cuticle					
Chitin	*Sig				
KOH Chitin	*Sig	*Sig			
BSA	*Sig	*Sig	*Sig		
Control	*Sig	*Sig	*Sig	*Sig	

3b) Vertalec 36 hours

F=88.06 P= <0.000 (4, 75 d.f)

	Cuticle	Chitin	KOH Chitin	BSA	Control
Cuticle					
Chitin	*Sig				
KOH Chitin	*Sig	*Sig			
BSA	*Sig	*Sig	*Sig		
Control	*Sig	*Sig	*Sig	*Sig	

4a) KV42 16 hours

F=57.05 P= <0.000 (4, 75 d.f.)

	Cuticle	Chitin	KOH Chitin	BSA	Control
Cuticle					
Chitin	Not sig.				
KOH Chitin	Not sig.	Not sig.			
BSA	*Sig	*Sig	*Sig		
Control	*Sig	*Sig	*Sig	*Sig	

4b) KV42 36 hours

F=30.82 P= <0.000 (d.f. 4, 75)

	Cuticle	Chitin	KOH Chitin	BSA	Control
Cuticle					
Chitin	Not sig.				
KOH Chitin	Not sig.	*Sig			
BSA	*Sig	*Sig	*Sig		
Control	*Sig	*Sig	*Sig	*Sig	

5a) KV22 16 hours

F=29.61 P= <0.000 (4, 75 d.f.)

	Cuticle	Chitin	KOH Chitin	BSA	Control
Cuticle					
Chitin	Not sig.				
KOH Chitin	Not sig.	Not sig.			
BSA	*Sig	*Sig	*Sig		
Control	*Sig	*Sig	*Sig	*Sig	

5b) KV22 36 hours

F=25.89 P= <0.000

	Cuticle	Chitin	KOH Chitin	BSA	Control
Cuticle					
Chitin	Not Sig.				
KOH Chitin	*Sig	*Sig			
BSA	*Sig	*Sig	*Sig		
Control	*Sig	*Sig	*Sig	*Sig	

2.2 CARBON AND NITROGEN DE/REPRESSION

1a) Mycotal 16 hours - CUTICLE

F=190.55 P= <0.000 (3, 60 d.f)

	-C-N	+C+N	+C-N	-C+N
-C-N				
+C+N	*Sig			
+C-N	*Sig	*Sig		
-C+N	*Sig	*Sig	Not sig.	

1b) Mycotal 36 hours - CUTICLE

F=188.20 P <0.000 (3, 60 d.f)

	-C-N	+C+N	+C-N	-C+N
-C-N				
+C+N	*Sig			
+C-N	*Sig	*Sig		
-C+N	*Sig	*Sig	Not sig	

2) KV54 36 hours - CUTICLE

F=41.83 P= <0.000 (3, 60 d.f.)

	-C-N	+C+N	+C-N	-C+N
-C-N				
+C+N	*Sig			
+C-N	*Sig	Not sig.		
-C+N	Not sig.	*Sig	*Sig	

3a) Vertalec 16 hours - CUTICLE

F=383.86 P= <0.000 (3, 60 d.f.)

	-C-N	+C+N	+C-N	-C+N
-C-N				
+C+N	*Sig			
+C-N	*Sig	*Sig		
-C+N	*Sig	*Sig	*Sig	

3b) Vertalec 36 hours - CUTICLE

F=232.91 P= <0.000 (3, 60 d.f.)

	-C-N	+C+N	+C-N	-C+N
-C-N				
+C+N	*Sig			
+C-N	*Sig	*Sig		
-C+N	*Sig	*Sig	*Sig	

4a) KV42 16 hours - CUTICLE

F=99.65 P= <0.000 (3, 60 d.f.)

	-C-N	+C+N	+C-N	-C+N
-C-N				
+C+N	*Sig			
+C-N	Not sig.	*Sig		
-C+N	Not sig.	*Sig	Not sig.	

4b) KV42 36 hours - CUTICLE

F=114.18 P= <0.000 (3, 60 d.f.)

	-C-N	+C+N	+C-N	-C+N
-C-N				
+C+N	*Sig			
+C-N	Not sig.	*Sig		
-C+N	Not sig.	*Sig	Not sig.	

5a) KV22 16 hours - CUTICLE

F=56.76 P= <0.000 (3, 60 d.f.)

	-C-N	+C+N	+C-N	-C+N
-C-N				
+C+N	*Sig			
+C-N	*Sig	*Sig		
-C+N	*Sig	*Sig	Not sig.	

5b) KV22 36 hours - CUTICLE

F=230.84 P= <0.000 (3,.60 d.f.)

	-C-N	+C+N	+C-N	-C+N
-C-N				
+C+N	*Sig			
+C-N	*Sig	*Sig		
-C+N	*Sig	*Sig	*Sig	

APPENDIX 3 - CALIBRATION OF PR1 ASSAY

Nmol nitroalanine released from the peptide substrate N-Suc-Ala-Ala-Pro-Phe-pNA was calculated from a calibration curve of known nitroalanine standards.

As calculated by Microsoft Excel: Intercept = -1.81

Slope = 337.13

RSQ = 0.98

Enzyme activity was converted to nkats by first converting to µmol NA, (x 1000) and then multiplying by 16.67. The amount of nitroalanine released per minute were expressed as that released per ml of enzyme.

APPENDIX 4 - ERGOSTEROL ASSAY

Ergosterol, as a measure of fungal biomass, was estimated for *V.lecanii* according to age of culture and used to compare transfer experiments. For full details please see J Graystone's thesis (University of Bath 1998).

Calibration of *V.lecanii* dry weights on days 3-6 of infection (Graystone 1998)

	Replicate 1 Dry weight	Replicate 1 Ergosterol	Replicate 2 Dry weight	Replicate 2 Ergosterol	Replicate 3 Dry weight	Replicate 3 Ergosterol
Day 3	0.73	600.00	0.62	489.00	0.46	598.00
Day 4	0.92	1160.00	0.82	1120.00	0.81	1201.00
Day 5	1.07	1600.00	1.11	1489.00	1.02	1456.00
Day 6	1.94	1698.00	1.54	1634.00	1.57	1621.00

	Av. Dry	Av. Ergost.	Yield per g	SD Yield	SD Dry	SD Ergost
Day 3	0.60	562.33	932.04	286.09	0.14	63.52
Day 4	0.85	1160.33	1365.10	110.98	0.06	40.50
Day 5	1.07	1515.00	1418.54	76.97	0.04	75.44
Day 6	1.68	1651.00	980.79	100.04	0.22	41.22

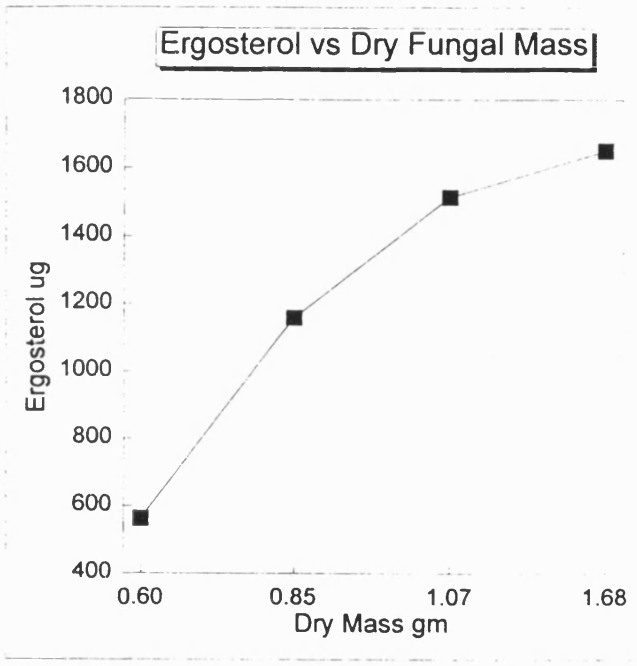

Fig. C/1

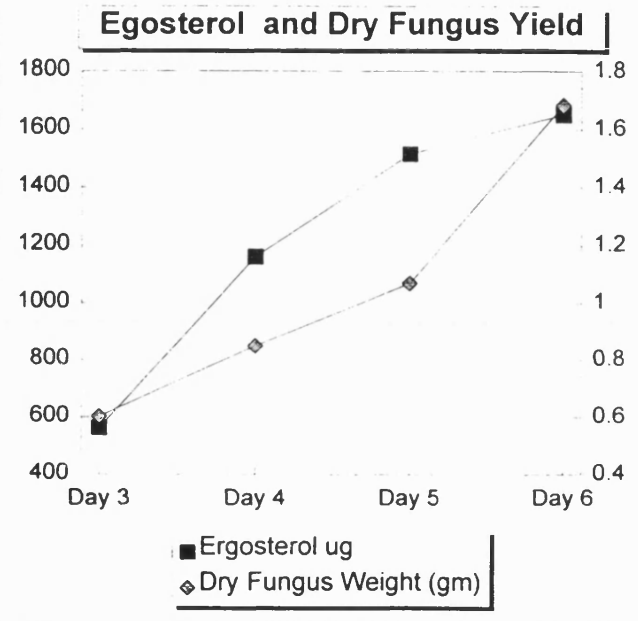

Fig. C/2

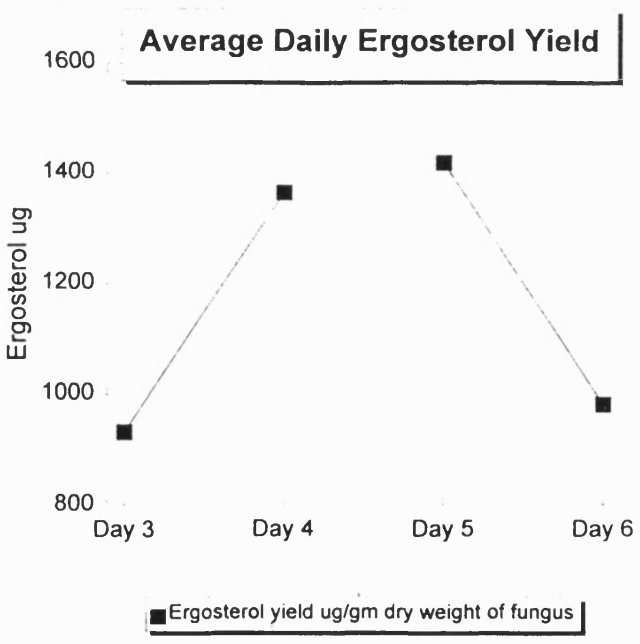

Fig. C/3